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INTRODUCTION

The research studies in this application proposes to determine if temporally regulated overexpression of TGF β 1 and TGF β 2 confers antiestrogen resistance to human breast cancer cells. In a naturally established model of tamoxifen resistance that overexpresses TGF β 2, we propose to determine if abrogation of TGF β 2 expression by genetic means restored tamoxifen action *in vivo*. Finally, the contribution of the host immune system to tamoxifen action will be explored.

BODY

Task 1. Generation of MCF-7 and T47D cells stably tranfected with TGF β 1 and TGF β 2 expression vectors. Unfortunately, we were unable to generate cells stably expressing regulatable tet-op/TGF β 1 or TGF β 2 expression vectors. We suspect that the reason was loss of the transactivating MMTV/tTA vector which has been shown to induce squelching of transcription factors and be toxic to some cells. Secondary attempts at transfecting constitutive CMV/TGF β 1 and CMV/TGF β 2 vectors resulted in cells with low levels of ectopic TGF β protein probably due to the inhibitory effect of TGF β *per se* on the tumor cells themselves.

Task 2. Development of antisense TGF β 2 vectors and generation of stably transfected LCC2 clones. LXS β N/AS-TGF β 2 was successfully transduced into LCC2 cells. However, it resulted in at best a 40-50% reduction in the level of TGF β 2 expression and secretion. It was our judgement that such a low level of reduction in TGF β 2 expression/secretion did not warrant use of these cells for studies in mice.

Tasks 3, 4, and 5. These sections of the proposal relied on the success of Tasks 1 and 2 and included experiments in mice to address tumorigenicity *in vivo* as well and determining the role of host natural killer (NK) cell function in tamoxifen action. Due to the problems in generating appropriate cell lines, these Tasks could not be completed.

As a result of these and consistent with the overall thematic of the grant proposal focused on the role of tumor cell TGF β s on the progression of breast cancer, we have pursued alternative studies to determine the TGF β -mediated signaling events leading to epithelial to mesenchymal transition, motility, and enhanced survival of mammary epithelial cells. Those studies are summarized below.

Phosphatidylinositol-3 kinase function is required for transforming growth factor β -mediated epithelial to mesenchymal transition and cell migration. We studied the role of signaling by phosphatidylinositol-3 kinase (PI3K) and its target, the serine/threonine kinase Akt, on TGF β -mediated epithelial to mesenchymal transition (EMT). In NMuMG mammary epithelial cells, exogenous TGF β 1 induced phosphorylation of Akt at Ser473 and Akt *in vitro* kinase activity against GSK-3 β within 30 min. These responses were temporally correlated with de-localization of E-cadherin, ZO-1 and integrin β 1 from cell junctions and the acquisition of spindle cell morphology. LY294002, an inhibitor of the p110 catalytic subunit of PI3K, and a dominant-negative mutant of Akt blocked the de-localization of ZO-1 induced by TGF β 1, whereas transfection of constitutively active p110 induced loss of ZO-1 from tight junctions. In addition, LY294002 blocked TGF β -mediated C-terminal phosphorylation of Smad2. Consistent with these data, TGF β -induced p3TP-Lux and p(CAGA)12-Lux reporter activities, were inhibited by LY294002 and transiently expressed dominant-negative p85 and Akt mutants in NMuMG and

4T1 cells. Dominant-negative RhoA inhibited TGF β -induced phosphorylation of Akt at Ser473 whereas constitutively active RhoA increased the basal phosphorylation of Akt, suggesting that RhoA is involved in TGF β -induced EMT. Finally, LY294002 and neutralizing pan-TGF β antibodies inhibited ligand-independent constitutively active Akt as well as basal and TGF β -stimulated migration in 4T1 and EMT6 breast tumor cells. Taken together, these data suggested for the first time that signaling by PI3K and Akt is required for TGF β -induced transcriptional responses, EMT, and migration of breast carcinoma cells.

Transforming growth factor β enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. The Forkhead family of transcription factors, which includes AFX, FKHR, and FKHRL1, participates in the induction of death-related genes. These factors contain classical Akt phosphorylation sites which, upon phosphorylation by Akt, result in their retention in the cytosol. In NMuMG and 4T1 mammary epithelial cells, TGF β induced phosphorylation and cytoplasmic retention of the Forkhead factor FKHRL1, while reducing FKHRL1-dependent transcriptional activity. TGF β -induced FKHRL1 phosphorylation and nuclear exclusion were inhibited by LY294002, an inhibitor of PI3K. A triple mutant of FKHRL1, in which all three Akt phosphorylation sites have been mutated (TM-FKHRL1), did not translocate to the cytoplasm in response to TGF β . In HaCaT keratinocytes, expression of dominant-negative Akt prevented TGF-induced 1) reduction of Forkhead-dependent transcription, 2) FKHRL1 phosphorylation, and 3) nuclear exclusion of FKHRL1. Forced expression of either wild-type (WT) or TM-FKHRL1, but not a FKHRL1 mutant with deletion of the transactivation domain, resulted in NMuMG mammary cell apoptosis. Evidence of nuclear fragmentation colocalized to cells with expression of WT- or TM-FKHRL1. The apoptotic effect of WT-FKHRL1 but not TM-FKHRL1 was prevented by exogenous TGF β . Serum starvation-induced apoptosis was also inhibited by TGF β in NMuMG and HaCaT cells. Finally, dominant-negative Akt abrogated the antiapoptotic effect of TGF β . Taken together, these data suggest that TGF β may play a role in epithelial cell survival via Akt-dependent regulation of FKHRL1. This report further highlights the role of Akt as a mediator of the tumor-promoting effects of the TGF β s.

p38 mitogen-activated protein kinase (p38Mapk) is required for TGF β -mediated fibroblastic transdifferentiation and cell migration. These studies further attempted to dissect the signaling programs regulated by TGF β that contributed to tumor cell motility and invasion. We found that TGF β -induced EMT was blocked by inhibiting activation of p38Mapk with H-7, a protein kinase inhibitor, or p38Mapk activity with SB202190, a p38Mapk inhibitor, suggesting involvement of p38Mapk in EMT. Inhibition of the p38Mapk pathway affected TGF β -mediated phosphorylation of ATF2, but did not inhibit phosphorylation of Smad2. SB202190 impaired TGF β -mediated changes in cell shape and reorganization of the actin cytoskeleton. Forced expression of dominant negative (DN) MKK3 inhibited TGF β -mediated activation of p38Mapk and EMT. Expression of DN-p38 α impaired TGF β -induced EMT. Inhibition of p38Mapk blocked TGF β -induced migration of non-tumor and tumor mammary epithelial cells. TGF β induced activation of the p38Mapk pathway within 15 min. Expression of TGF β type II (T β RII) and type I (T β RI/Alk5) kinase-inactive receptors blocked EMT and activation of p38Mapk, whereas expression of constitutively active Alk5-T204D resulted in EMT and phosphorylation of MKK3/6 and p38Mapk. Finally, dominant negative Rac1N17 blocked TGF β -induced activation of the p38Mapk pathway and EMT, suggesting that Rac1 mediates activation of the p38Mapk

pathway. These studies suggest that the p38Mapk pathway is required for TGF β -mediated EMT and cell migration.

Smad signaling is not sufficient for TGF β -mediated motility in human cancer cells. There is increasing evidence that after cells lose their sensitivity to TGF β -mediated growth inhibition, autocrine TGF β signaling may potentially promote tumor cell motility and invasiveness. A key question of high therapeutic relevance is whether the effects of TGF β on epithelial tumor cell plasticity and cancer progression require the function of the Smad signal transducers. In order to address this question, we have generated MDA-MB-231 human breast cancer cells stably expressing a kinase-inactive T β RII (T β RII-K277R). Our data indicate that T β RII-K277R is expressed, can associate with the type I TGF β receptor, and block both Smad-dependent and -independent signaling pathways activated by TGF. In addition, wound closure and transwell migration assays indicated that the basal migratory potential of T β RII-K277R expressing cells is impaired. The impaired motility of T β RII-K277R cells could be restored by reconstituting TGF β signaling with a constitutively active TGF β type I receptor (Alk5), suggesting that the impairment of tumor cell motility is Alk5-specific. However, reconstitution of Smad signaling by forced expression of Smad2/4 or Smad3/4, at levels resulting in high TGF β reporter activity, failed to restore motility of tumor cells expressing the dominant negative T β RII. Likewise, re-expression of Smad4 in both the Smad4 defective MDA-MB-468 breast cancer cells and the SW480.7 colorectal cancer cells, did not promote tumor cell migration. These data suggest that Smad signaling alone is not sufficient to restore TGF β -mediated motility in cancer cells in which T β RII signaling is blocked nor to reconstitute TGF β -mediated motility in Smad4-null tumor cells.

KEY RESEARCH ACCOMPLISHMENTS

The key research accomplishments can be summarized as it follows:

1. PI3K, Akt, and p38Mapk are part of a signaling program upregulated by TGF β that is required to induce tumor cell motility, invasion, and metastases.
2. TGF β can induce Akt-dependent FKHRL1 phosphorylation, resulting in enhanced mammary cell survival and potentially contributing further to tumor progression.
3. Blockade of autocrine TGF β signaling in human breast cancer cells impedes tumor cell motility which can not be restored by forced expression of Smad proteins. Smad proteins alone are unable to reconstitute TGF β -mediated motility of human cancer cells.

REPORTABLE OUTCOMES (reprints enclosed)

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol-3 kinase function is required for TGF β -mediated epithelial to mesenchymal transition and cell migration. *J. Biol. Chem.* 275:36803-36810, 2000

Shin I, Bakin AV, Rodeck U, Brunet A, Arteaga CL. Transforming growth factor β enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. *Mol. Biol. Cell* 12:3328-3339, 2001

Bakin AV, Rinehart CC, Tomlinson AK, Arteaga CL. p38 mitogen-activated protein kinase is required for transforming growth factor β -mediated fibroblastic transdifferentiation and cell migration. *J. Cell Science* (In press), 2002

Dumont N, Bakin AV, Arteaga CL. Smad signaling is not sufficient for TGF β -mediated motility in human cancer cells. Under review (*J. Biol. Chem.*)

CONCLUSIONS

The overall conclusions from the body of work supported by the DAMD are summarized above under key research accomplishments. Overall, they suggest several key signaling pathways usurped by TGF β in human breast cancer in order to induce enhanced tumor cell survival, progression, and metastases. These pathways do not include the Smad signal transducers and represent rational targets for novel therapeutic strategies in breast cancer.

Transforming Growth Factor β Enhances Epithelial Cell Survival via Akt-dependent Regulation of FKHRL1

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The Forkhead family of transcription factors participates in the induction of death-related genes. In NMuMG and 4T1 mammary epithelial cells, transforming growth factor β (TGF β) induced phosphorylation and cytoplasmic retention of the Forkhead factor FKHRL1, while reducing FKHRL1-dependent transcriptional activity. TGF β -induced FKHRL1 phosphorylation and nuclear exclusion were inhibited by LY294002, an inhibitor of phosphatidylinositol-3 kinase. A triple mutant of FKHRL1, in which all three Akt phosphorylation sites have been mutated (TM-FKHRL1), did not translocate to the cytoplasm in response to TGF β . In HaCaT keratinocytes, expression of dominant-negative Akt prevented TGF β -induced 1) reduction of Forkhead-dependent transcription, 2) FKHRL1 phosphorylation, and 3) nuclear exclusion of FKHRL1. Forced expression of either wild-type (WT) or TM-FKHRL1, but not a FKHRL1 mutant with deletion of the transactivation domain, resulted in NMuMG mammary cell apoptosis. Evidence of nuclear fragmentation colocalized to cells with expression of WT- or TM-FKHRL1. The apoptotic effect of WT-FKHRL1 but not TM-FKHRL1 was prevented by exogenous TGF β . Serum starvation-induced apoptosis was also inhibited by TGF β in NMuMG and HaCaT cells. Finally, dominant-negative Akt abrogated the antiapoptotic effect of TGF β . Taken together, these data suggest that TGF β may play a role in epithelial cell survival via Akt-dependent regulation of FKHRL1.

INTRODUCTION

Transforming growth factor β (TGF β) is involved in various cellular processes, including cell division, differentiation, motility, adhesion, and apoptosis (Massagué, 1998). TGF β stimulates the proliferation of mesenchymal cells while inhibiting the growth of most normal epithelial cells (Massagué and Chen, 2000; Massagué and Wotton, 2000). TGF β signals are transmitted through a heterodimeric complex of two transmembrane serine/threonine kinases, the type I and

II TGF β receptors (Massagué, 1998; Massagué and Wotton, 2000). Receptor-associated Smads are intracellular signal transducers that associate with T β RI, become phosphorylated, and translocate to the nucleus where they regulate transcription of TGF β target genes (Lagna *et al.*, 1996; Massagué and Chen, 2000). TGF β modulates several signaling pathways in mammalian cells. The c-Jun NH₂-terminal kinase (JNK) can either be activated (Atfi *et al.*, 1997) or inhibited by TGF β (Imai *et al.*, 1999; Huang *et al.*, 2000). Rapid activation of extracellular signal-regulated kinase by TGF β has been reported in epithelial cells (Hartsough *et al.*, 1996). We previously reported that TGF β phosphorylates Akt in a phosphatidylinositol-3 kinase (PI3K)-dependent manner, response that was required for TGF β -mediated epithelial-to-mesenchymal transition and migration of mammary cells (Bakin *et al.*, 2000).

The Akt kinase is activated by phosphorylation at Thr308 and Ser473 mediated by 3-phosphoinositide-dependent protein kinase 1 and 2, respectively (Alessi *et al.*, 1997;

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Abbreviations used: dn, dominant-negative; FasL, Fas ligand; FCS, fetal calf serum; FHRE, Forkhead response element; JNK, c-Jun NH₂-terminal kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3 kinase; T β RI, TGF β type I receptor; Tet, tetracycline; TGF β , transforming growth factor β ; TM, triple mutant; WT, wild-type.

Stokoe *et al.*, 1997; Stephens *et al.*, 1998). Akt phosphorylates and inactivates glycogen synthase kinase3- β , an enzyme that regulates glycogen biosynthesis (Cross *et al.*, 1995). In addition to regulating cellular metabolism, Akt can promote enhanced cell survival (Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Datta *et al.*, 1999). Bad was the first reported proapoptotic factor directly phosphorylated and inactivated by Akt (del Peso *et al.*, 1997). Akt also phosphorylates the proapoptotic molecule caspase 9 at Ser196 (Cardone *et al.*, 1998), which results in suppression of caspase 9-induced apoptosis in 293 cells (Datta *et al.*, 1999).

The role of Akt in gene transcription was first discovered by studies performed in *Caenorhabditis elegans*. DAF-16, a Forkhead transcription factor in *C. elegans*, is negatively regulated by Akt. DAF-16 is activated by DAF-2 and DAF-23, where DAF-2 is an insulin receptor-like protein, and DAF-23, a PI3K-like protein (Kops and Burgering, 1999). The mammalian orthologs of DAF-16 are AFX, Forkhead response element (FKHR), and FKHRL1. In *C. elegans*, mutations of *daf2* synergize with mutations of *daf1*, a type I TGF β receptor, in inducing dauer formation (Ogg *et al.*, 1997), suggesting that TGF β can interact with the DAF-2/DAF-16 pathway. All three DAF-16 mammalian homologs share a Forkhead 100-amino acid core domain, responsible for binding to DNA. AFX, FKHR, and FKHRL1, each contain three Akt phosphorylation sites (Datta *et al.*, 1999), which can be phosphorylated by Akt in mammalian cells (Brunet *et al.*, 1999; Guo *et al.*, 1999; Kops *et al.*, 1999; Nakae *et al.*, 1999). On phosphorylation by Akt, Forkhead factors translocate from the nucleus to the cytoplasm (Biggs *et al.*, 1999; Brunet *et al.*, 1999), where 14-3-3 proteins may sequester them and prevent their function (Brunet *et al.*, 1999). In their unphosphorylated state, Forkhead factors predominantly localize in the nucleus where they bind to insulin response elements and/or the Fas ligand (FasL) promoter and activate transcription of target genes that may induce cell death (Brunet *et al.*, 1999; Kops and Burgering, 1999; Tang *et al.*, 1999). Accordingly, overexpression of either FKHR or FKHRL1 results in apoptosis (Brunet *et al.*, 1999; Tang *et al.*, 1999). So far, FasL is the only known candidate gene to mediate FKHRL1-induced apoptosis (Brunet *et al.*, 1999). Therefore, by phosphorylating FKHRL1 and excluding it from the nucleus, the PI3K target Akt may prevent the transcriptional engagement of FKHRL1, inhibit Forkhead-induced apoptosis, and contribute to cell survival.

In this report, we show that treatment with TGF β results in phosphorylation and nuclear exclusion of endogenous and ectopic FKHRL1 in mammary epithelial cells and skin keratinocytes. This effect required PI3K and Akt function as inhibitors of PI3K or expression of dominant-negative Akt (dn Akt) prevented TGF β -mediated inhibition of FKHRL1. Moreover, both Forkhead-dependent transcription and cell death induced by either serum starvation or forced expression of FKHRL1 was partially blocked by TGF β . These results suggest that TGF β , via activation of Akt, may induce cytoplasmic retention of FKHRL1 and thus act as an antiapoptotic factor in epithelial cells. These mechanisms may be biologically relevant to TGF β -mediated tumor progression.

MATERIALS AND METHODS

Cell Lines, Inhibitors, and Antibodies

NMuMG nontumorigenic mouse mammary epithelial cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 μ g/ml insulin. 4T1 breast tumor cells, kindly provided by F. Miller (Karmanos Cancer Center, Detroit, MI), were maintained in DMEM with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. HaCaT keratinocytes, stably transfected with a dn Akt mutant vector or vector alone (mock cells), have been described previously (Jost *et al.*, 2001). The HaCaT cells were maintained in DMEM supplemented with 10% FCS, 0.1 mg/ml hygromycin B, 2 μ g/ml tetracycline (Tet), 100 U/ml penicillin, and 100 μ g/ml streptomycin. To induce expression of the dn Akt protein, cells were washed two times with Tet-free medium and kept in Tet-free medium for 48 h. TGF β 1 was obtained from R & D Systems (Minneapolis, MN). Antibodies to FKHRL1, phospho-Ser-253 FKHRL1 and C-terminal phospho Smad2 were from Upstate Biotechnology (Lake Placid, NY) to Smad2 from Santa Cruz Biotechnology (Santa Cruz, CA). Texas Red-conjugated anti-mouse IgG secondary antibody was purchased from Molecular Probes (Eugene, OR) and anti-hemagglutinin (HA)-fluorescein mouse monoclonal antibody from Roche Molecular Biochemicals (Indianapolis, IN). Akt and phospho-Ser-473 Akt polyclonal antibodies were from New England Biolabs (Beverly, MA). LY294002 was from Calbiochem (San Diego, CA).

Immunoblot Analysis

After washes with phosphate buffered saline (PBS), cell monolayers were lysed in a buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, and 2 μ g/ml leupeptin. Equal amount of protein in whole cell lysates [as measured by Bradford (1976) method] were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in Tris-buffered saline-Tween 20 (TBST) containing 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20 (v/v) for 1 h at ambient temperature and then incubated overnight with primary antibodies in TBST in 1% skim milk at 4°C. After washing membranes with TBST three times, they were incubated with a 1:5000 dilution of horseradish peroxidase-linked secondary antibody in TBST for 1 h, followed by three washes in TBST. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce Chemical, Rockford, IL).

Immunocytochemistry and Transfections

Cells were grown in DMEM/10% FCS to ~60% confluence on glass coverslips in 12-well plates, washed with serum-free medium, incubated in serum-free medium for 24 h, and then stimulated with 2 ng/ml TGF β for 1 h in the absence or presence of 20 μ M LY294002, an inhibitor of PI3K (Vlahos *et al.*, 1994). In experiments involving ectopic FKHRL1 expression, cells in 60-mm dishes (10⁶ cells/dish) were transfected with 10 μ g of either WT-FKHRL1 or triple mutant (TM)-FKHRL1, each for 16 h with the use of FUGENE 6 (Roche Molecular Biochemicals). Cells were then transferred to coverslips on 12-well plates, incubated in serum-free medium for 24 h, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Coverslips were next blocked with 3% skim milk in PBS for 30 min and incubated with primary antibodies diluted in 1% skim milk/PBS (1:500 for FKHRL1 and P-Ser253 FKHRL1; 1:200 for anti-HA fluorescein). After three washes with PBS, samples were incubated with fluorescent secondary antibodies diluted in PBS (1:500) for 1 h at room temperature. Coverslips were mounted on glass slides with AquaPolyMount (Polysciences, Warrington, PA) and examined by laser scanning confocal microscopy (LSM 410;

Carl Zeiss, Thornwood, NY). For detection of apoptotic nuclei, cells were incubated in 1 μ g/ml Hoechst 33258 (Sigma, St. Louis, MO) in PBS for 10 min after incubation with secondary antibody. Fluorescent images of Hoechst-stained nuclei or HA-stained samples were recorded with a Zeiss Axiophot upright microscope.

Transcriptional Reporter Assays

Cells were seeded at the density of 10^5 cells/well (12-well plates). After 24 h, the cells were transfected with 0.5 μ g/well of either WT-FKHRL1-HA or TM-FKHRL1-HA, each with 0.5 μ g/well of Forkhead-responsive element (FHRE)-Luc and 0.005 μ g/well of pCMV-Rl (Promega, Madison, WI) with the use of 3 μ l/well of FUGENE6 reagent for 16 h. Transfected cells were then subjected to serum starvation either in the presence or absence of 2 ng/ml TGF β for 24 h. Firefly luciferase and *Renilla reniformis* luciferase activities in cell lysates were determined with the use of the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol in a Monolight 2001 luminometer (Analytical Luminescence Laboratory). Firefly luciferase activity was normalized to *R. reniformis* luciferase activity and presented as relative luciferase units.

Apoptosis Assays

Cells were seeded at the density of 5×10^5 cells/well in six-well dishes. The following day, the medium was changed to serum-free medium with or without 2 ng/ml TGF β . Both floating cells and adherent cells were harvested 72 h later. Pooled cells were washed with PBS and then subjected to Apo-5-bromo-2'-deoxyuridine (BrdU) analysis with the use of an Apo-BrdU assay kit (Phoenix Flow Systems, San Diego, CA) according to the manufacturer's protocol in a FACS/Calibur Flow Cytometer (BD Biosciences, Mansfield, MA). For evaluation of DNA fragmentation, 10^6 cells/dish in 60-mm dishes were incubated in serum-free medium \pm 2 ng/ml TGF β . After 72 h, floating and adherent cells were harvested, washed with PBS, and resuspended in 200 μ l of cytosolic DNA extraction buffer (5 mM Tris, pH 7.4, 20 mM EDTA, 0.5% Triton X-100) followed by vortexing for 1 min. After centrifugation for 15 min at 12,000 rpm at 4°C, the supernatants were transferred into new tubes and subjected to phenol/chloroform extraction. The DNA fragments were pelleted by adding 3 M sodium acetate, washed with ethanol, resuspended in TE containing 200 μ g/ml RNase, and separated by 1.5% agarose gel electrophoresis.

Cell Cycle Analysis by Flow Cytometry

Cells were harvested by trypsinization, fixed in ethanol, and labeled with 50 μ g/ml propidium iodide (Sigma) containing 125 U/ml protease-free RNase (Calbiochem) as described previously (Busse *et al.*, 2000). Cells were filtered through a 95- μ m pore size nylon mesh (Small Parts, Miami Lakes, FL) and a total of 15,000 stained nuclei was analyzed in a FACS/Calibur Flow Cytometer (BD Biosciences).

RESULTS

TGF β Induces Phosphorylation and Nuclear Exclusion of Endogenous FKHRL1

We have previously observed that TGF β can phosphorylate and activate Akt (Bakin *et al.*, 2000), which is known to phosphorylate FKHRL1 both in vivo and in vitro (Brunet *et al.*, 1999). Thus, we first determined whether TGF β can induce FKHRL1 phosphorylation. As shown in Figure 1A, treatment with 2 ng/ml TGF β for 30 min increased the phosphorylation of FKHRL1 in 4T1 and NMuMG cells as determined by immunoblot with a P-Ser253 FKHRL1 antibody. Akt can phosphorylate FKHRL1 at Thr32, Ser253, and Ser315. Brunet *et al.* (1999) showed that the Akt-dependent

shift in FKHRL1 mobility on SDS-PAGE is primarily due to phosphorylation at Ser315, suggesting that the slower migrating band in the lysates from TGF β -treated cells (Figure 1A) may represent P-Ser315 FKHRL1 also recognized by the P-Ser253 FKHRL1 antibody. Cotreatment with LY294002 inhibited TGF β -mediated phosphorylation of FKHRL1, suggesting that this effect required PI3K function.

Others have reported that Forkhead transcription factors translocate from the nucleus to the cytoplasm after Akt-mediated phosphophorylation (Biggs *et al.*, 1999; Brunet *et al.*, 1999; del Peso *et al.*, 1999; Takaishi *et al.*, 1999). To determine whether TGF β can induce translocation of endogenous FKHRL1, we stimulated serum-starved cells with 2 ng/ml TGF β for 30 min and then performed immunofluorescence analysis. Figure 1B shows FKHRL1 staining in cytoplasm and nucleus in both NMuMG and 4T1 cells. TGF β treatment results in exclusion of the nuclear FKHRL1 staining, which was prevented by LY294002. These results suggest that TGF β -mediated phosphorylation and subsequent nuclear exclusion of FKHRL1 are both PI3K-dependent.

TGF β Inhibits Nuclear Translocation of Exogenous FKHRL1 and Forkhead-dependent Transcription

We next determined whether TGF β -mediated FKHRL1 regulation required function of the PI3K effector kinase Akt. Cells were transfected with HA-tagged WT-FKHRL1 or a triple mutant TM-FKHRL1, in which all three Akt phosphorylation sites (Thr32, Ser253, and Ser315) (Brunet *et al.*, 1999) had been mutated to alanine, and examined the subcellular localization of ectopic FKHRL1 under various conditions. In serum-containing medium and in both 4T1 and NMuMG mammary cells, WT-FKHRL1 primarily localized in the cytoplasm (Figure 2A, first column of each cell line). On removal of serum for 24 h, WT-FKHRL1 protein localized predominantly in the nucleus. Treatment with TGF β promoted WT-FKHRL1 translocation to the cytoplasm, which was prevented by cotreatment with the PI3K inhibitor LY294002. 4T1 and NMuMG cells transfected with the TM-FKHRL1 construct exhibited exclusive nuclear localization of the HA-tagged mutant under any experimental condition (Figure 2A, third row). The results imply that the phosphorylation status of the three Akt sites determines the subcellular distribution of FKHRL1: unphosphorylated FKHRL1 is localized mainly in the nucleus; phosphorylation of the three Akt-consensus sites upon the addition of TGF β results in nuclear exclusion of FKHRL1.

We next asked whether Forkhead-dependent transcription was regulated by TGF β signaling. We cotransfected cells with either a WT- or TM-FKHRL1 constructs and a FHRE-Luc vector in which a Forkhead response element is linked to a luciferase reporter gene. FHRE contains the binding site of the FasL promoter and FKHRL1 is known to bind this site and enhance transcription of the FasL gene (Brunet *et al.*, 1999). Withdrawal of serum increased FKHRL1-dependent luciferase expression in both NMuMG and 4T1 cells (Figure 2B). However, the addition of TGF β reduced luciferase activity to levels obtained in the presence of serum, suggesting that TGF β down-regulates Forkhead-dependent transcription possibly by a mechanism involving nuclear exclusion of FKHRL1. In cells transfected with the Akt-insensitive TM-FKHRL1, transcriptional activity was higher

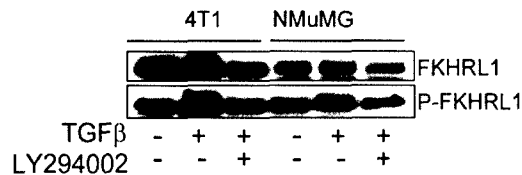
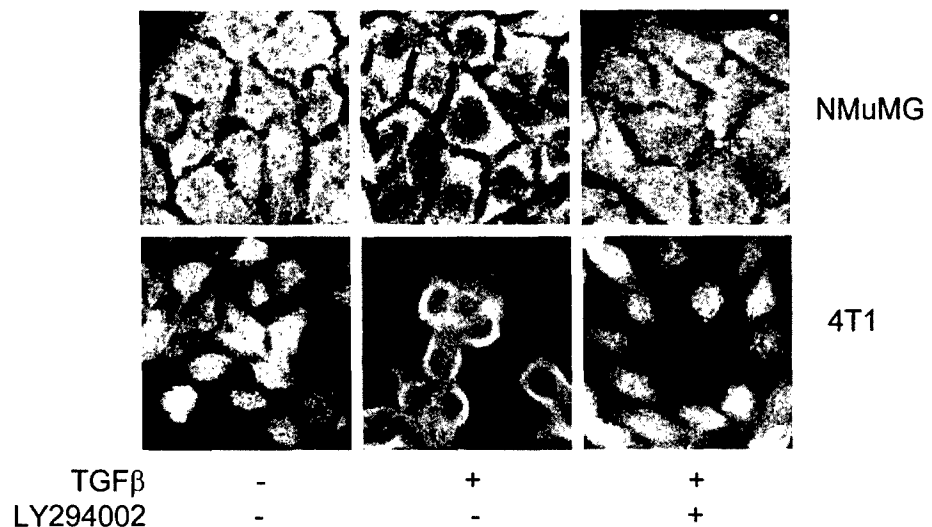
A

Figure 1. TGF β induces cytoplasmic retention and phosphorylation of FKHRL1 in Ser253. (A) Serum-starved, subconfluent 4T1 and NMuMG cells were treated with 2 ng/ml TGF β for 30 min with or without 20 μ M LY294002. After lysis, protein extracts (50 μ g/lane) were subjected to SDS-PAGE followed by immunoblot procedures as described in MATERIALS AND METHODS. Dilution ratio for primary antibodies is 1:500 for both FKHRL1 and P-Ser253 FKHRL1. (B) 4T1 and NMuMG cells grown to 60% confluence on coverslips in 12-well plates, serum-starved for 24 h, and then treated with 2 ng/ml TGF β for 30 min with or without 20 μ M LY294002. Cells were washed, fixed as described in MATERIALS AND METHODS, and stained with antibodies against FKHRL1 (1:500). Texas Red-conjugated anti-rabbit IgG (1:500) was used as secondary antibody. Fluorescent images were captured with the use of a laser scanning confocal microscope.

B

than in cells transfected with WT-FKHRL1. Although to a lesser degree than in cells transfected with the WT construct, TGF β also reduced the transcriptional activity mediated by TM-FKHRL1 (Figure 2B).

Dominant-Negative Akt Inhibits TGF β -induced FKHRL1 Phosphorylation, Nuclear Exclusion of FKHRL1, and Forkhead-dependent Transcription

The causal role of Akt in TGF β -mediated regulation of FKHRL1 was investigated with the use of HaCaT keratinocytes expressing Tet-suppressible dn Akt (Jost *et al.*, 2001). The construct used in this system encodes a kinase-inactive version of Akt in which Lys179 in the catalytic domain has been mutated to Met (Dudek *et al.*, 1997). Withdrawal of Tet from the cell culture medium for 48 h induced expression of dn Akt (Figure 3A). Treatment with TGF β also induced Akt activity, as measured by P-Ser473 Akt and P-Ser253 FKHRL1 immunoblot analyses in both mock HaCaT cells \pm Tet and in dn Akt HaCaT cells in the presence of Tet (Figure 3A). However, when the dn Akt transgene was expressed by withdrawing Tet from dn Akt HaCaT cell culture medium for 48 h, TGF β -induced phosphorylation at Ser253 of FKHRL1, Ser473 of Akt (Figure 3A) were all reduced. Consistent with transcriptional reporter activity data from NMuMG and 4T1 cells, in mock-

transfected HaCaT cells \pm Tet and in dn Akt HaCaT cells treated with Tet, TGF β reduced FKHRL1-dependent transcription (Figure 3B, left). In dn Akt HaCaT cells, however, removal of Tet blocked the inhibitory effect of added TGF β on FKHRL1-induced reporter activity (Figure 3B, right), suggesting that Akt is indeed responsible for mediating this TGF β response. To exclude the possibility that dn Akt may be blocking TGF β effects on FKHRL1 via inhibition of the TGF β type I receptor (T β RI) kinase, we tested the effect of dn Akt on phosphorylation of Smad2. As shown in Figure 3C, TGF β -mediated C-terminal phosphorylation of Smad2 in HaCaT cells was not altered by dn Akt.

Immunocytochemical analysis (Figure 4) shows that induction of dn Akt can block nuclear exclusion of exogenously expressed FKHRL1 in HaCaT keratinocytes. In mock HaCaT cells, serum starvation resulted in nuclear localization of WT-FKHRL1; treatment with TGF β for 30 min promoted translocation of WT-FKHRL1 from the nucleus to the cytosol regardless of the presence of Tet. Expression of kinase-inactive Akt by withdrawal of Tet from culture medium, blocked the ability of TGF β to induce nuclear exclusion of WT-FKHRL1. As expected, TM-FKHRL1 localized in the nucleus of both dn Akt and control cells regardless of the presence of Tet (Figure 4).

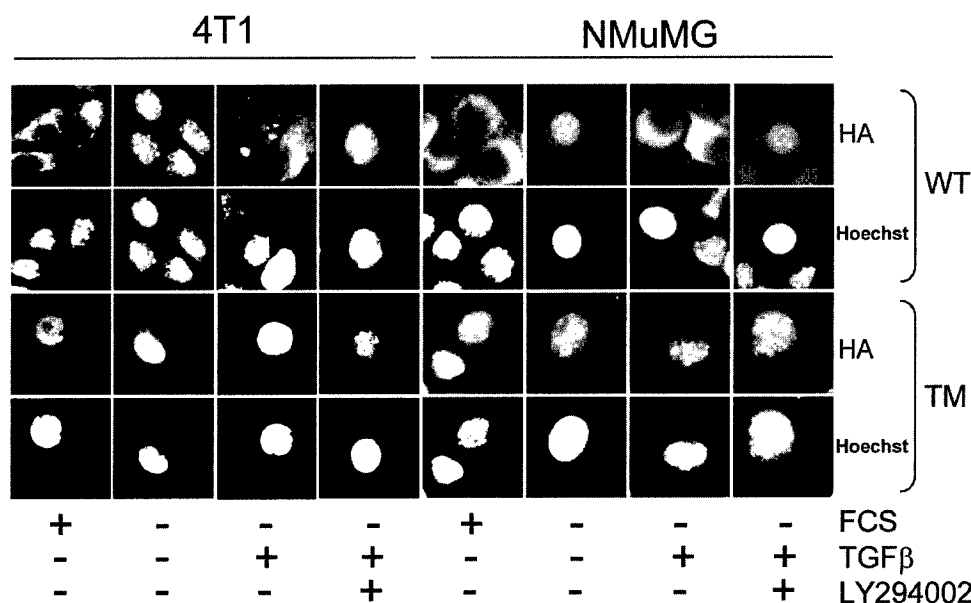
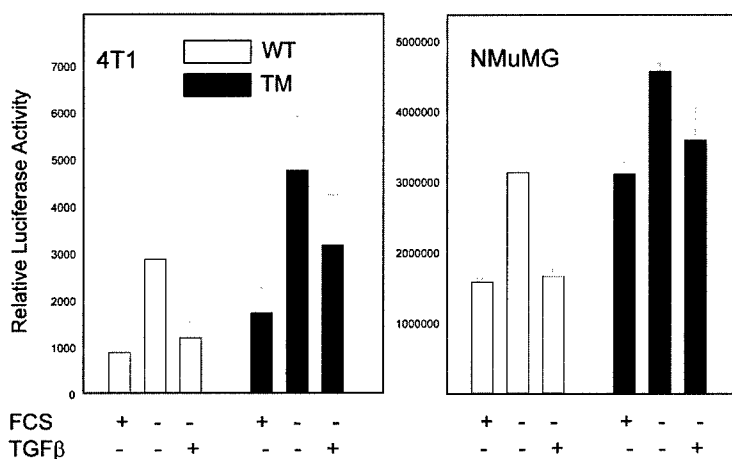
A

Figure 2. TGF β inhibits nuclear translocation of exogenous FKHRL1 and Forkhead-dependent transcription. (A) Cells seeded in 60-mm dishes (10^6 cells/dish) were transfected with 10 μ g/dish of either WT- or TM-FKHRL1-HA for 16 h and transferred to coverslips on 12-well plates. The cells were then incubated in serum-free medium for 24 h followed by stimulation with 2 ng/ml TGF β for 30 min with or without 20 μ M LY294002. Fixation and staining were performed as described in MATERIALS AND METHODS. Fluorescence intensities associated with anti-HA fluorescein and Hoechst 33258 were observed with a Zeiss Aioptot upright microscope. (B) Cells were transfected with WT- or TM-FKHRL1, each with FHRE-Luc and pCMV-Rl vectors followed, where indicated, with removal of serum and the addition or not of 2 ng/ml TGF β for 24 h. Dual luciferase assay was performed as described in MATERIALS AND METHODS. Relative luciferase units represents firefly luciferase activity normalized to *Renilla* luciferase activity. Each data point represents mean \pm SD of four wells.

B

TGF β Partially Suppresses Apoptosis Induced by Serum Starvation or by Forced Expression of WT-FKHRL1 but not by TM-FKHRL1

Because TGF β can induce phosphorylation and subsequent nuclear exclusion of FKHRL1, which is known to be responsible for the expression of death-related genes (Brunet *et al.*, 1999; Kops and Burgering, 1999), we investigated whether expression of ectopic FKHRL1 and/or serum starvation can induce apoptosis in NMuMG cells and whether TGF β can reverse this process. As shown in Figure 5A, forced expression of either WT- or TM-FKHRL1 resulted in NMuMG cell apoptosis. Cells transfected with either construct were double-labeled with the nuclear stain Hoechst 33258 and an HA antibody. The same cells expressing WT- or TM-FKHRL1, as measured by HA staining, exhibited fragmented nuclei, sug-

gesting that FKHRL1 causes NMuMG cell death. Of \sim 150 HA-positive nuclei, 45% of WT-FKHRL1- and 67% TM-FKHRL1-expressing nuclei were apoptotic.

To further determine whether nuclear localization of FKHRL1 was causal to apoptosis, we examined the temporal correlation of nuclear localization of FKHRL1 with the onset of DNA double-strand breaks as measured by Apo-BrdU assay. Initial experiments showed that \geq 2 μ g/ml exogenous WT-FKHRL1 was required to induce apoptosis of NMuMG cells (our unpublished results). Therefore, to minimize a possible contribution of the ectopic FKHRL1 to NMuMG cell apoptosis, we used a >10 -fold lower concentration (0.2 μ g/ml) of HA-tagged WT-FKHRL1. Nuclear localization of FKHRL1 was first evident at 3 h after serum starvation, whereas a low level

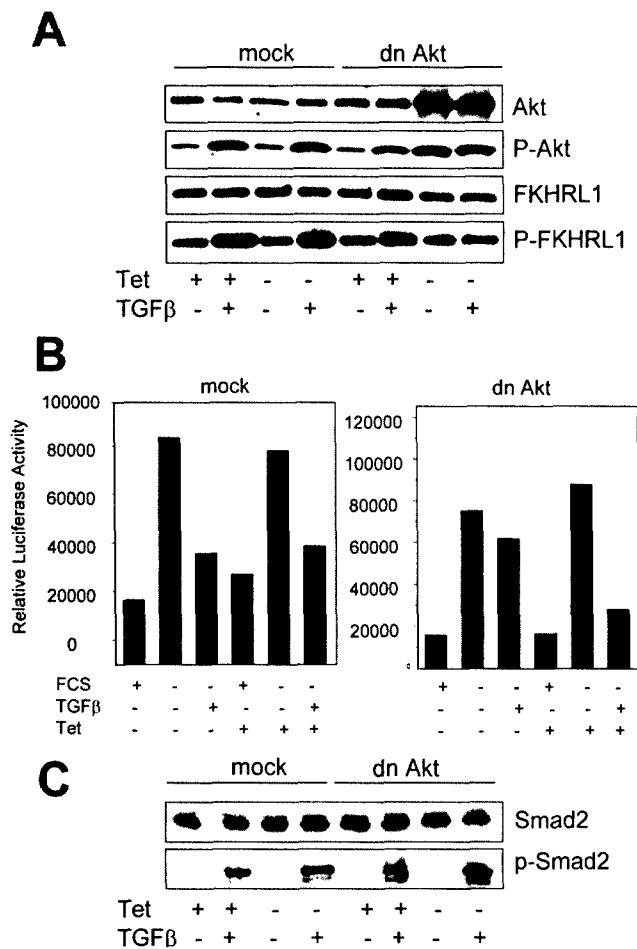


Figure 3. Dominant-negative Akt blocks TGF β -induced FKHRL1 phosphorylation and Forkhead-dependent transcription but not phosphorylation of Smad2. (A) Mock-transfected and dn Akt-transfected HaCaT cells were incubated in medium with or without 2 μ g/ml Tet for 48 h. For the last 24 h of incubation, medium was replaced by serum-free medium \pm Tet. The cells were then treated with 2 ng/ml TGF β for 30 min, harvested, and lysed. Protein extracts (30 μ g/lane) were subjected to SDS-PAGE followed by immunoblot as described in MATERIALS AND METHODS. Dilution ratios for primary antibodies are 1:500 for P-Ser473 Akt, FKHRL1, and P-Ser253 FKHRL1, and 1:1000 for total Akt. (B) HaCaT cells were transfected with WT- or TM-FKHRL1, each with FHRE-Luc and pCMV-Rl vectors, and incubated in medium with or without 2 μ g/ml Tet for 48 h. For the last 24 h where indicated, medium was replaced with serum-free DMEM \pm Tet with or without 2 ng/ml TGF β . Dual luciferase assay was performed as described in MATERIALS AND METHODS. Each data point represents mean \pm SD of four wells. (C) In the presence or absence of Tet, cells were incubated with 2 ng/ml TGF β for 30 min and lysed as in Figure 3A. Cell lysates were resolved by SDS-PAGE and subjected to immunoblot procedures for total and phosphorylated Smad2. Each lane contains 50 μ g of protein. Dilution ratios for primary antibodies are 1:1000 for total Smad2 and 1:500 for P-Smad2.

of apoptosis above baseline was first detectable at 12 h reaching a maximum at 48 h, implying that the nuclear localization of FKHRL1 was not secondary to the onset of apoptosis.

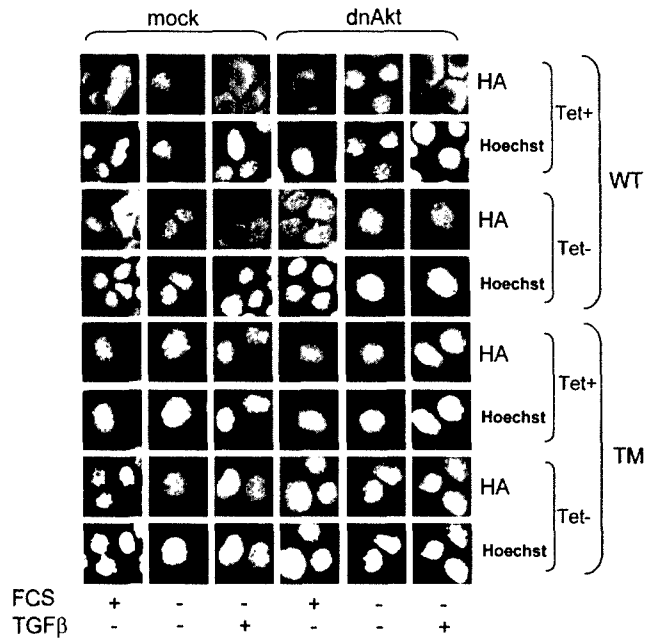


Figure 4. Dominant-negative Akt blocks TGF β -induced nuclear exclusion of FKHRL1. Mock and dn Akt HaCaT cells seeded in 60-mm dishes (10^6 cells/dish) \pm Tet were transfected with 10 μ g/dish of either WT- or TM-FKHRL1 for 16 h and next transferred to coverslips on 12-well plates. In some cases, the cells were serum-starved for 24 h followed by the addition of 2 ng/ml TGF β for 30 min. Fixation and staining were performed as described in MATERIALS AND METHODS. Fluorescence intensities associated with anti-HA fluorescein and Hoechst 33258 were measured as described in the legend of Figure 2A.

We next investigated whether addition of exogenous TGF β could rescue apoptosis induced by serum starvation or forced expression of FKHRL1. Removal of FCS for 72 h increased the proportion of apoptotic cells from 7.98 to 46.78%. The latter was reduced to 24.07% by the addition of 2 ng/ml TGF β (Figure 6A). Transfection of 5 μ g/ml WT- or TM-FKHRL1 increased the proportion of apoptotic cells from 8.60% to 25.66 or 46.86%, respectively (Figure 6B). Notably, expression of FKHRL1 mutant constructs with a deletion of the transactivating Forkhead domain (FKHRL1 Δ TA and TM-FKHRL1 Δ TA) did not induce apoptosis above baseline, implying that the transactivating function of FKHRL1 was required for the induction of cell death. Addition of TGF β markedly inhibited the apoptosis induced by WT-FKHRL1 but not by TM-FKHRL1 (Figure 6B), further suggesting that the protective effect of TGF β depended on Akt-mediated phosphorylation of FKHRL1.

Dominant-Negative Akt Abolishes Survival Effect of TGF β

To obtain direct evidence that Akt may play a role in TGF β -induced cell survival, we used HaCaT cells in which dn Akt was conditionally expressed. As measured by Apo-BrdU assay, a large proportion of mock-transfected HaCaT cells (>20%) became apoptotic upon withdrawal of serum for 72 h. In the presence or absence of Tet, exogenous TGF β

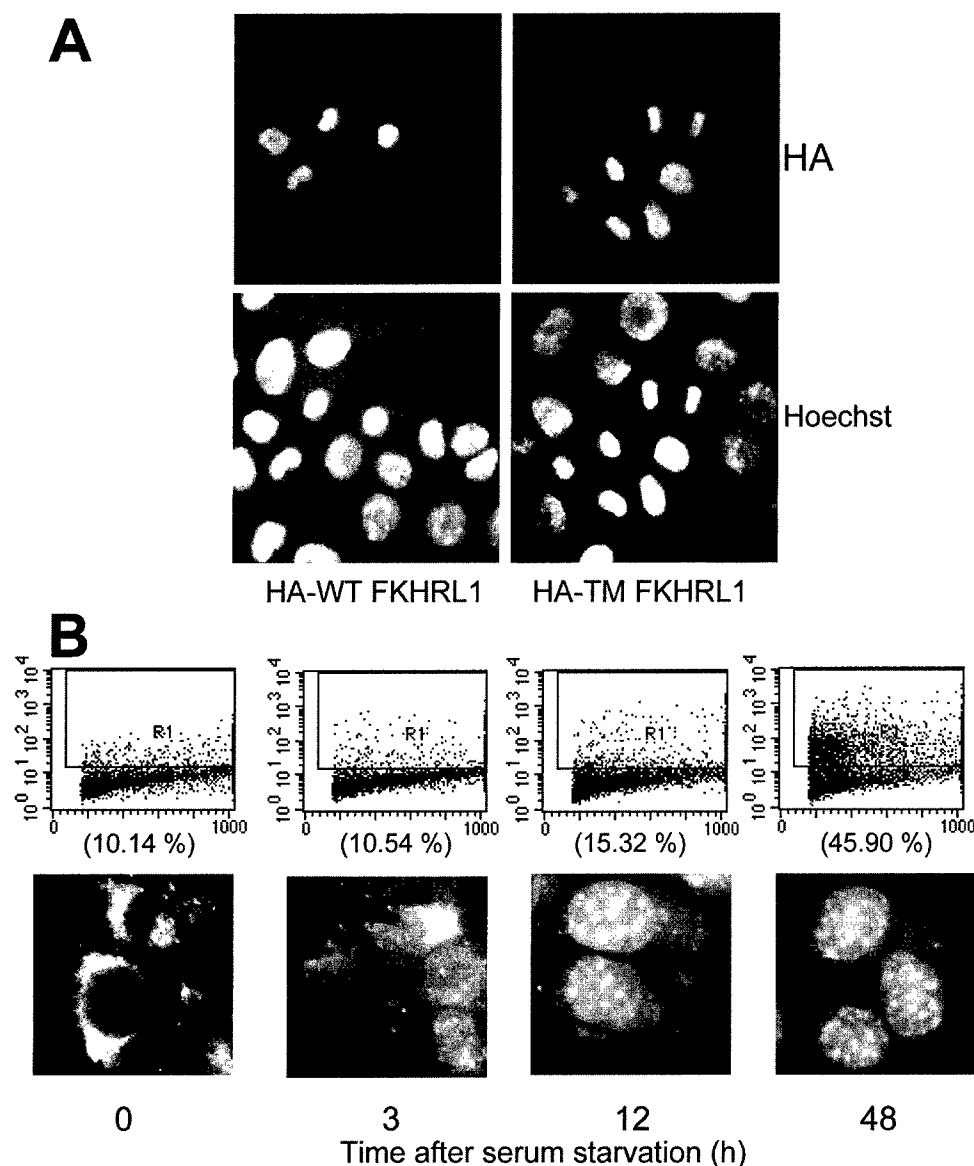


Figure 5. Forced expression of FKHL1 or serum starvation induce apoptosis of NMuMG cells. (A) NMuMG cells in 60-mm dishes (10^6 cells/dish) were transfected with 10 μ g/dish of either WT- or TM-FKHL1-HA for 16 h and transferred to coverslips on 12-well plates. After 24-h incubation, the cells were stained with anti-HA fluorescein and Hoechst 33258. The samples were examined in a Zeiss Axiophot microscope. (B) NMuMG cells in 60-mm dishes (10^6 cells/dish) were transfected with 0.2 μ g/ml WT-FKHL1-HA for 16 h and transferred to coverslips on 12-well plates. After 24 h, the medium was changed with serum-free medium. At the indicated times, the cells were harvested and assayed for evidence of apoptosis by Apo-BrdU analysis (top row) or stained with anti-Ha fluorescein and observed with a Zeiss Axiophot microscope. The percentages of fluorescein isothiocyanate-positive (apoptotic) cells are shown in parentheses.

completely prevented apoptosis in control (mock) HaCaT cells and in dn Akt cells treated with Tet (Figure 7A, second column). On the other hand, in dn Akt HaCaT cells, removal of Tet and hence induction of kinase-dead Akt, blocked the ability of TGF β to prevent the apoptosis induced by serum starvation. Similar data were obtained in DNA fragmentation assays. Serum starvation induced internucleosomal DNA fragmentation in both control and dn Akt cells. TGF β abolished DNA fragmentation except in dn Akt cells in the absence of Tet (Fig. 7B), implying Akt is causal to the protection from cell death mediated by TGF β .

Survival Effects of TGF β Are Independent of Antiproliferative Effects

Addition of TGF β to proliferating NMuMG cells induces growth arrest. These studies have been done with exponen-

tially growing NMuMG cells in serum-containing medium (Miettinen *et al.*, 1994; Piek *et al.*, 1999), conditions under which FKHL1 localizes mainly in the cytosol and FKHL1-mediated transcription is low (Figure 2). In serum-containing medium, NMuMG displayed a robust S phase (24.3%) and a low level of apoptosis. A 24-h treatment with TGF β resulted in G1 arrest and marked reduction in S phase without a significant effect on the low level of apoptosis (6.9 vs. 11.2%; Figure 8A). Under these serum-containing conditions, TGF β induced a 44% reduction in cell number after 72 h (Figure 8B), consistent with the delay in cell cycle progression. On the other hand, serum withdrawal (for 24 h) *per se* resulted in G1 arrest (89.2%), whereas 21.9% of cells exhibited evidence of apoptosis. Addition of TGF β reduced in half the apoptosis induced by serum deprivation (21.9 vs. 10.8%) but had no detectable effect on NMuMG cell cycle

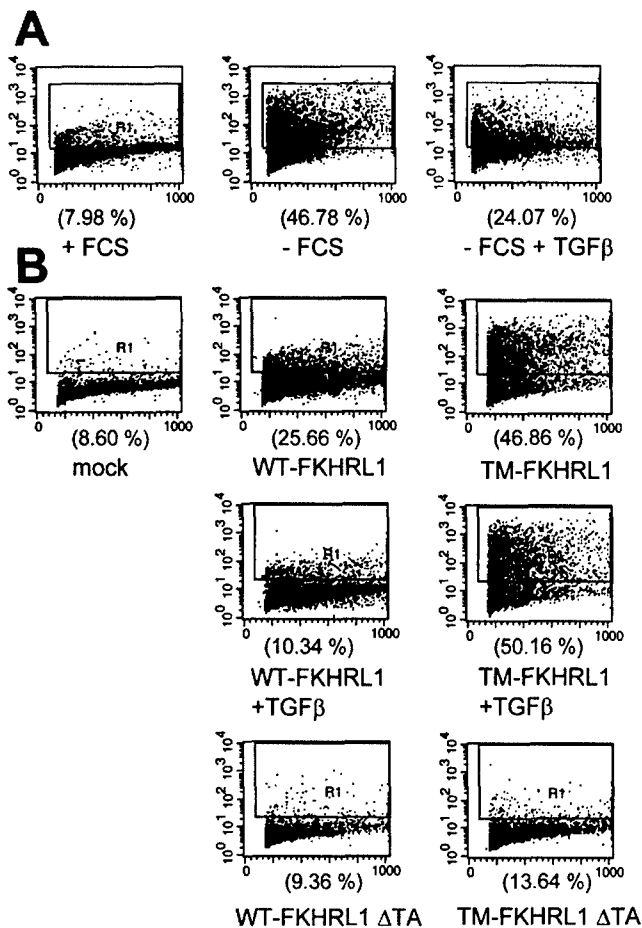


Figure 6. TGF β inhibits NMuMG cells apoptosis induced by serum starvation or by forced expression of FKHRL1. (A) NMuMG cells seeded in serum-free medium at the density of 5×10^5 cells/well in six-well dishes were treated with or without 2 ng/ml TGF β . Seventy-two hours later, the cells were harvested and evaluated for apoptosis by Apo-BrdU analysis as described in MATERIALS AND METHODS. (B) NMuMG cells seeded at the density of 5×10^5 cells/well in six-well dishes were transfected with 5 μ g/ml the indicated WT- and TM-FKHRL1 constructs for 16 h. After transfection, cells were incubated for 72 h in the presence or absence of 2 ng/ml TGF β and finally subjected to Apo-BrdU analysis.

distribution as measured by flow cytometry (Figure 8A). Consistent with its antiapoptotic effect, these results, addition of TGF β to serum-deprived cells increased cell number 90% above untreated control cells after 72 h (Figure 8B). These data suggest that TGF β -mediated signals that result in growth inhibition may be independent from those involved in the blockade of apoptosis and enhanced survival.

DISCUSSION

Results presented herein with mammary epithelial cells and skin keratinocytes indicate that TGF β induced phosphorylation and nuclear exclusion of both the endogenous and transfected Forkhead transcription factor FKHRL1.

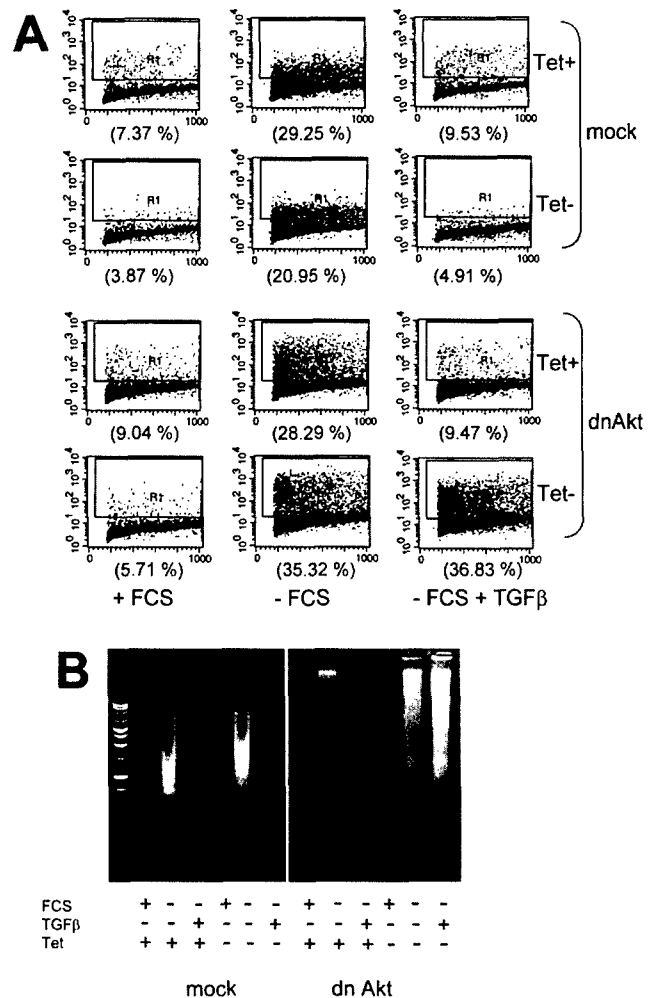


Figure 7. Expression of dominant-negative Akt blocks the antiapoptotic effect of TGF β . (A) Control (mock) and dn Akt HaCaT keratinocytes were incubated in serum-containing or serum-free medium \pm Tet for 72 h in the presence or absence of 2 ng/ml TGF β . The proportion of apoptotic cells was assessed by Apo-BrdU analysis and is indicated in parentheses. (B) Mock and dn Akt HaCaT cells (10^6 cells/dish) on 60-mm dishes were incubated in medium containing FCS or in serum-free medium \pm 2 ng/ml TGF β for 72 h. To suppress dn Akt, Tet was added during the incubation period where indicated. Adherent and floating cells were pooled, their DNA was collected, and next evaluated for evidence of internucleosomal fragmentation in 1.5% agarose gels as described in MATERIALS AND METHODS.

LY294002, a small molecule inhibitor of the p110 catalytic subunit of PI3K, blocked these effects, suggesting that TGF β -mediated regulation of FKHRL1 required PI3K function. LY294002 did not inhibit TGF β -induced PAI-1 luciferase reporter activity in Mink lung epithelial cells (our unpublished data). This suggests that the blockade of TGF β effects on FKHRL1 by LY294002 was not due to inhibition of TGF β type I receptor (T β RI) kinase activity. Moreover, dn Akt did not prevent TGF β -induced C-terminal phosphorylation of Smad2 in HaCaT cells (Figure 3C) nor in NMuMG cells

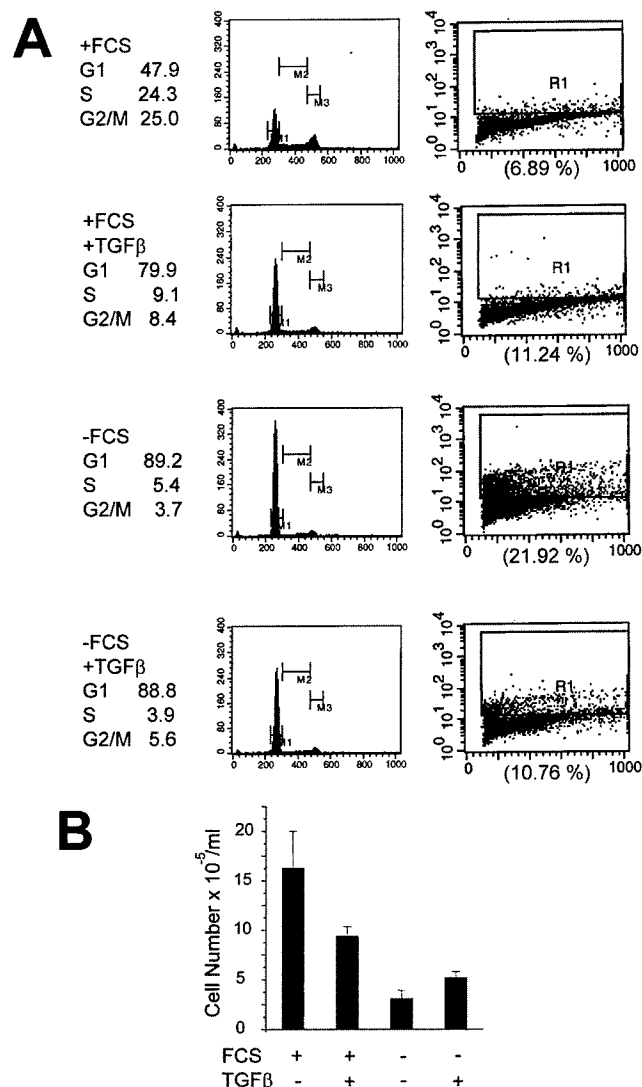


Figure 8. TGF β inhibits cell cycle progression of proliferating NMuMG cells but protects serum-starved NMuMG cells from apoptosis. (A) NMuMG cells seeded at the density of 10^6 cells/dish in 60-mm dishes were incubated in DMEM/10% FCS or serum-free DMEM, each \pm 2 ng/ml TGF β . After 24 h, cells were harvested and analyzed for cell cycle and apoptosis as described in MATERIALS AND METHODS. (B) NMuMG cells (10^6 cells/dish) in 60-mm dishes were incubated in DMEM/10% FCS or serum-free DMEM, each \pm 2 ng/ml TGF β for 72 h. At this time, the monolayers were washed with PBS and the adherent cells were trypsinized and counted with the use of a Coulter counter. Each bar represents the mean \pm SD of four dishes.

(Bakin *et al.*, 2000), implying further that blockade of TGF β action on FKHRL1 by dn Akt did not involve an effect on T β RI kinase activity.

Several results implicated activation of Akt as an effector mechanisms for FKHRL1 regulation by TGF β . First, a mutant of Forkhead in which all three Akt phosphorylation sites have been replaced with Ala localized exclusively in

the nucleus of three cell lines (NMuMG, 4T1, and HaCaT) and was insensitive to TGF β -mediated retention in the cytosol. Second, in HaCaT cells, inducible kinase-inactive Akt blocked the effects of TGF β on FKHRL1 Ser253 phosphorylation, nuclear exclusion, and transcriptional activity. Third, in serum-starved NMuMG and 4T1 cells, the transcriptional activity of TM-FKHRL1 was higher than that of WT-FKHRL1. However, the TM-FKHRL1-mediated transcription was still inhibited by TGF β , suggesting that the mutant construct was unable to override endogenous Forkhead factors potentially regulated by the addition of TGF β . Thus, we speculate that the reduction in reporter activity mediated by TGF β in TM-FKHRL1-expressing cells (Figure 2B) might be conferred by the endogenous FKHRL1, which is still subjected to Akt-mediated phosphorylation. Finally, the apoptotic effect of WT-but not TM-FKHRL1 was abrogated by TGF β as long as Akt was functional. Nonetheless, transfection of a FKHRL1 constructs in which the DNA-binding domain was mutated (H212R) did not prevent TGF β -induced protection from cell death (our unpublished data). The inability of this ectopic protein with intact Akt phosphorylation sites to reduce the antiapoptotic effect of TGF β implied that Akt-mediated phosphorylation of FKHRL1 may not be a saturable process. Although the induced dn Akt was effective in blocking TGF β -induced nuclear exclusion and cytosolic retention of FKHRL1, it only partially induced nuclear localization of FKHRL1 in the presence of serum without added TGF β (Figure 4). It is conceivable that other activities not affected by dn Akt, such as serum- and glucocorticoid-induced kinases (SGKs; Brunet *et al.* 2001), may also regulate FKHRL1 localization and/or function in these cells, potentially explaining the cytosolic retention of FKHRL1 despite expression of dn Akt shown in Figure 4. This possibility will require further experiments beyond the scope of this report.

The potent antiapoptotic effect of Akt and its disabling effect of FKHRL1 function suggested that FKHRL1 may induce apoptosis in the epithelial cells used in our studies. Indeed, overexpression of both WT- and TM-FKHRL1 induced NMuMG cell death as implied by nuclear fragmentation in cells also expressing either the ectopic WT- or TM-FKHRL1 construct (Figure 5A). Apoptosis was not observed with transient transfection of a mutant FKHRL1 that lacked DNA-transactivating function. Notably, transient transfection efficiencies were consistently low (\approx 3%) with the WT and TM constructs but much higher (\approx 15%) with FKHRL1 Δ TA, further supporting a causal association between FKHRL1 expression and apoptosis. Moreover, the nuclear localization of FKHRL1 in serum-starved NMuMG cells preceded the onset of apoptosis, implying that cell death is subsequent to the nuclear localization of FKHRL1 and that the latter was not due to an indirect effect of the apoptotic program on nuclear export. Supporting this possibility is the fact that, despite inducing high levels of apoptosis, TM-FKHRL1 was consistently localized in the nucleus (Figures 2A, 4, and 5A). Consistent with the transcriptional reporter activity driven by an FHRE (Figure 2B), the proportion of apoptotic cells was higher in NMuMG cells transfected with TM- than with WT-FKHRL1 (Figure 5A; see RESULTS), implying that a low level of basal Akt activity is able to ameliorate the cell death induced by WT-FKHRL1. It is conceivable that inhibition of other proapop-

otic molecules beyond the scope of this report, such as BAD, caspase 9, or I κ Ks (Datta *et al.*, 1999), are involved in Akt-mediated protection from cell death. However, in preliminary experiments we have been unable to detect increased caspase 9 activity in serum-starved NMuMG cells.

Our data concur with those in other reports. Tang *et al.* (1999) showed that another Forkhead ortholog (FKHR), in which all three Akt phosphorylation sites have been mutated to Ala (TM-FKHR), induced features of apoptosis as membrane blebbing and DNA fragmentation 48 h after transfection into 293T cells. Cells transfected with WT-FKHR showed minimal evidence of apoptosis despite expressing higher level of WT-FKHR than TM-FKHR. In addition, a mutation in the DNA-binding domain of FKHR reduced the ability of this expression vector to induce apoptosis (Tang *et al.*, 1999). In this report (Figure 6B), a mutant FKHL1 lacking its transactivation domain failed to elicit apoptosis, implying that both DNA binding and activation of transcription are required for the occurrence of Forkhead-induced apoptosis. Brunet *et al.* (1999) reported induction of apoptotic cell death by a triple-Akt-sites-mutant of FKHL1 in cerebellar neurons, CCL39 fibroblasts, and Jurkat T cells. They also provided some evidence that FKHL1-induced apoptosis was mediated in part by its ability to induce transcription of the FasL gene. Recently, it was reported that TGF β could decrease apoptosis of human T cells while inhibiting the expression of FasL (Genestier *et al.*, 1999). In PC12 cells, removal of nerve growth factor results in increased JNK activation, enhanced FasL expression, and neuronal cell death (Le-Niculescu *et al.*, 1999). In addition, studies in T lymphocytes have shown that forced expression of FKHL1 up-regulates the anti-Bcl-2 molecule Bim concomitant with the induction of apoptosis (Dijkers *et al.*, 2000b). Both Forkhead factors AFX and FKHL1 also have been shown to induce transcription of the cyclin-dependent kinase inhibitor p27^{Kip1} (Dijkers *et al.*, 2000a). This allows for effectors of PI3K, via phosphorylation of Forkhead factors and inhibition of p27 gene transcription, to regulate cell proliferation in addition to cell survival. However, because we did not observe changes in cell cycle distribution in TGF β -protected cells, which would have been expected from down-regulation of p27, we did not pursue the role of this cyclin-dependent kinase inhibitor on TGF β -mediated enhanced survival.

The effect of TGF β on apoptosis has been investigated in different cell systems. In some cells, TGF β is a potent inducer of apoptosis (Selvakumaran *et al.*, 1994; Lomo *et al.*, 1995; Sánchez *et al.*, 1996), whereas in others it can effectively inhibit apoptosis (Sachsenmeier *et al.*, 1996; Chin *et al.*, 1999; Saile *et al.*, 1999; Huang *et al.*, 2000). In human keratinocytes, the apoptotic cell death induced by loss of anchorage is attenuated by both endogenous and exogenous TGF β (Sachsenmeier *et al.*, 1996). In this study, TGF β -neutralizing antibodies enhanced DNA fragmentation after cell suspension, indicating that endogenous TGF β , via autocrine signaling, may mediate the enhanced survival. The apoptosis precipitated by serum starvation in macrophages is also prevented by exogenous TGF β via mitogen-activated protein kinase (Chin *et al.*, 1999). In A549 lung adenocarcinoma cells, the antiapoptotic effect of TGF β requires modulation of JNK activity and phosphorylation of c-Jun (Huang *et al.*, 2000). In addition, TGF β was reported to act as a survival

factor to prevent c-Myc induced cell death in Rat-1 fibroblasts and this response was independent of any effect on cell cycle progression. Expression of dominant-negative forms of various components of the JNK signaling pathway, including Rac1, Cdc42, MKK4, and c-Jun abolished TGF β -induced survival (Mazars *et al.*, 2000). In our studies with NMuMG cells, the survival effect of TGF β was clearly dissociated from its antimitogenic effect (Figure 8). Although it is still conceivable that the same Smad-mediated transcriptional responses that induce epithelial cell cytoskeleton mediate the antiapoptosis effect of TGF β , a requirement of Smad signaling for the regulation of FKHL1 and the prevention of cell death in epithelial cells requires further investigation. In a recent study, however, overexpression of Smad2 almost completely abrogated the JNK-dependent survival effect of TGF β (Mazars *et al.*, 2000), suggesting that Smad signaling was independent and antagonistic of the latter cellular response.

In summary, our results suggest that FKHL1-dependent transcription may play a role in inducing apoptotic epithelial cell death and that TGF β partially reverses this effect by mechanism(s) involving Akt-dependent phosphorylation and nuclear exclusion of FKHL1. We recently reported that PI3K function and its effector kinase Akt are required for TGF β -mediated fibroblastic transition and cell migration in epithelial cells (Bakin *et al.*, 2000), events involved in the metastatic progression of carcinomas. Transfection of dominant-negative T β RII constructs that disable autocrine TGF β have been shown to inhibit this mesenchymal transdifferentiation and reduce tumor cell invasiveness and metastases (Oft *et al.*, 1998; Portella *et al.*, 1998; Yin *et al.*, 1999; McEarchern *et al.*, 2001) suggesting that via subversion of an epithelial phenotype, autocrine/paracrine TGF β can contribute to the metastatic progression of epithelial cancers. Based on the data presented, we propose that down-regulation of Forkhead-dependent transcription and its subsequent positive effect on epithelial cell survival is an integral part of a multisignaling program by which TGF β contributes to epithelial transformation and tumor progression.

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Phosphatidylinositol 3-Kinase Function Is Required for Transforming Growth Factor β -mediated Epithelial to Mesenchymal Transition and Cell Migration*

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We have studied the role of phosphatidylinositol 3-OH kinase (PI3K)-Akt signaling in transforming growth factor β (TGF β)-mediated epithelial to mesenchymal transition (EMT). In NMuMG mammary epithelial cells, exogenous TGF β 1 induced phosphorylation of Akt at Ser-473 and Akt *in vitro* kinase activity against GSK-3 β within 30 min. These responses were temporally correlated with delocalization of E-cadherin, ZO-1, and integrin β_1 from cell junctions and the acquisition of spindle cell morphology. LY294002, an inhibitor of the p110 catalytic subunit of PI3K, and a dominant-negative mutant of Akt blocked the delocalization of ZO-1 induced by TGF β 1, whereas transfection of constitutively active p110 induced loss of ZO-1 from tight junctions. In addition, LY294002 blocked TGF β -mediated C-terminal phosphorylation of Smad2. Consistent with these data, TGF β -induced p3TP-Lux and p(CAGA)₁₂-Lux reporter activities were inhibited by LY294002 and transiently expressed dominant-negative p85 and Akt mutants in NMuMG and 4T1 cells. Dominant-negative RhoA inhibited TGF β -induced phosphorylation of Akt at Ser-473, whereas constitutively active RhoA increased the basal phosphorylation of Akt, suggesting that RhoA is involved in TGF β -induced EMT. Finally, LY294002 and neutralizing TGF β 1 antibodies inhibited ligand-independent constitutively active Akt as well as basal and TGF β -stimulated migration in 4T1 and EMT6 breast tumor cells. Taken together, these data suggest that PI3K-Akt signaling is required for TGF β -induced transcriptional responses, EMT, and cell migration.

The transforming growth factor β (TGF β)¹ family of secreted

factors is involved in the control of different biological processes including cell proliferation, differentiation, and apoptosis (1). TGF β signals through the activation of heteromeric complexes of TGF β type I (T β RI) and type II (T β RII) receptors (1, 2). Activated T β RI phosphorylates receptor-associated Smads (Smad2 and Smad3), which then bind Smad4 and translocate to the nucleus where they regulate transcription of target genes (3, 4). TGF β exhibits a tumor suppressor activity, and components of its signaling pathway are frequently mutated or silenced in colon and pancreatic cancers (1, 5). However, accumulating data indicate that TGF β can positively affect tumorigenesis and contribute to the progression and invasiveness of tumors (5–8). Moreover, it was recently reported that inhibition of autocrine TGF β signaling in carcinoma cells reduces cell invasiveness and tumor metastases (9, 10). These effects of TGF β are associated with its ability to induce an epithelial to mesenchymal transition (EMT) and stimulate cell migration.

The EMT induced by TGF β results in the disruption of the polarized morphology of epithelial cells, formation of actin stress fibers, and enhancement of cell migration (8, 9). Two species of T β RI, Alk2 and Alk5, have been implicated in the induction of EMT by TGF β in mammary epithelial cells (11, 12). It has also been reported that high levels of ectopic Smad2 and Smad3 can induce some features of EMT in mammary epithelial cells in the context of expression of an activated type I receptor (12). However, considering the complexity of TGF β signaling (3, 13–16), it is conceivable that other molecules can also contribute to EMT. For example, members of the AP-1 family of transcription factors have been shown to induce EMT and promote tumor invasiveness (17, 18). AP-1 complexes can be activated in response to TGF β (19–21), physically interact with Smads (13, 14), and cooperate with Smads in the control of gene expression (19–21). In addition, several other downstream signaling pathways can also be activated by TGF β , including p38Mapk (21), c-jun N-terminal kinase (22, 23), and phosphatidylinositol 3-OH kinase (PI3K) (24, 25). These signaling pathways can potentially contribute to TGF β 1-mediated EMT, but their significance for EMT and cell migration mediated by TGF β remains unclear.

In this study, we used the NMuMG mammary epithelial cell line as a model for TGF β 1-induced EMT (11). Two metastatic breast tumor cell lines, 4T1 and EMT6, that express high levels of TGF β ligands and TGF β receptors were used in transcription and migration studies. We report that TGF β -induced EMT

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¹ The abbreviations used are: TGF β , transforming growth factor β ; T β RI, TGF β type I; EMT, epithelial to mesenchymal transition; PI3K, phosphatidylinositol 3-OH kinase; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered

saline; MT, microtubule; ca, constitutively active; dn, dominant-negative; PKB, protein kinase B; SARA, Smad activator for receptor activation; FYVE domain, domain found in Fab1p, YOTB, Vac1p, and EEA1 proteins.

and cell migration depend on the PI3K-Akt pathway. We also show that the phosphorylation of Smad2 and transcriptional responses induced by TGF β are inhibited by pharmacological and molecular antagonists of the PI3K-Akt pathway. TGF β 1 can induce phosphorylation and activation of Akt/PKB in a PI3K-dependent manner, and this activation requires the Rho GTPase function. Taken together, our data suggest that PI3K-Akt signaling is required for the morphogenic, transcriptional, and migratory activities of TGF β .

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—TGF β 1 was from R & D Systems (Minneapolis, MN) and EGF from CLONTECH (Palo Alto, CA). Antibodies to E-cadherin and integrin β_1 were from Transduction Laboratories (Lexington, KY), to p85 from Upstate Biotechnology (Lake Placid, NY), and to ZO-1 from Chemicon (Temecula, CA). Phalloidin-fluorescein isothiocyanate (actin) was from Molecular Probes (Eugene, OR). The TGF β 1-neutralizing 2G7 monoclonal IgG₂ was a gift from B. Fendly (Genentech, Inc.) and has been described previously (26). Antibodies to phospho-Ser-473 Akt and total Akt were from New England BioLabs (Beverly, MA), to Smad2 (N19) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and to C-terminal phospho-Smad2 from Upstate Biotechnology. Antibodies to phospho-ERK1/2 and total ERK1/2 were from Promega (Madison, WI) and New England BioLabs, respectively. Mouse monoclonal antibodies 12CA5 and M2 to HA and Flag epitopes were from Roche Molecular Biochemicals and Sigma, respectively. Anti-Myc mouse monoclonal 9E10 antibody was a gift from J. F. Primus (Vanderbilt University). LY294002, ML7, okadaic acid, PD098059, rapamycin, U0126, and U73122 were purchased from Calbiochem (San Diego, CA). Curcumin was from Sigma. The Rac1 inhibitor SCH51344 was a kind gift from C. Kumar (Schering Research Institute, Kenilworth, NJ) (27). Adenovirus vectors encoding a dominant-negative mutant of Akt (AxAktK179D), a mutant regulatory subunit of p85 (AxAp85), and a constitutively active myristoylated mutant of p110 (AxMyr-p110) were kindly provided by W. Ogawa (Kobe University School of Medicine, Kobe, Japan) (28). The pCMV6-AktK179M mutant was a gift from P. N. Tsichlis (Thomas Jefferson University, Philadelphia, PA). Plasmid vectors encoding Q61LRhoA and N19RhoA mutants were obtained from Dr. Lynn Cross (National Institutes of Health, Bethesda, MD). A plasmid vector encoding a GST-GSK3 β peptide fusion protein was a gift from C. L. Van Den Berg (University of Colorado, Denver).

Cell Culture and Adenoviral Infection—NMuMG cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FBS and 10 μ g/ml insulin. 4T1 tumor cells were provided by F. Miller (Karmanos Cancer Center, Detroit, MI) and EMT6 tumor cells by B. Teicher (Lilly Research Laboratories, Indianapolis, IN); both were cultured in DMEM plus 10% FBS. For adenoviral infection of NMuMG and 4T1 cells, 10⁵ cells/well in 6-well plates were transduced with adenovirus vectors at 10–100 plaque-forming units/cell as described by Sakaue *et al.* (29). More than 90% of the NMuMG cells infected at a similar multiplicity of infection with an adenovirus expressing β -galactosidase (Ad β -Gal) exhibited blue staining. Infected cells were subjected to further treatment 24–48 h later.

Cell Lysis and Immunoblot Analysis—Cells were lysed in EBC buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, and 2 μ g/ml leupeptin), and protein concentrations in cell lysates were determined by the Bradford method. Protein extracts (50 μ g/lane) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (100 mA, 2.5 h). Membranes were blocked with 5% milk in TBST buffer (containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20 (v/v)) for 1 h at room temperature and then incubated with primary antibodies in TBST plus 1% milk for 16 h at 4 °C followed by incubation with secondary antibodies for 1 h at room temperature. Membranes were washed three times in TBST and immunoreactive bands visualized by ECL (Pierce).

Akt/PKB *In Vitro* Kinase Assay—Akt/PKB was precipitated from protein extracts (150 μ g) with GST-GSK-3 β fusion protein immobilized on agarose beads (Sigma) or GST-agarose beads for 2 h at 4 °C. An *in vitro* kinase reaction was performed by adding 10 μ Ci of [γ -³²P]ATP (specific activity, 3000 Ci/mmol; PerkinElmer Life Sciences) for 20 min at 30 °C in the presence of 10 μ M PKA peptide inhibitor (Calbiochem). Reaction was terminated by the addition of 5 \times Laemmli buffer and heating followed by 15% SDS-PAGE. Quantitative analysis of ³²P-

labeled bands was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Transcriptional Assays—NMuMG, 4T1, and EMT6 cells (0.5 \times 10⁶) were seeded in 60-mm dish and transfected the following day with 0.5 μ g/ml p3TP-Lux (provided by J. Massague, Memorial Sloan-Kettering Cancer Center, New York, NY) or p(CAGA)₁₃-Lux (provided by J.-M. Gauthier, Laboratoire Glaxo Wellcome, Les Ulis Cedex, France), each with 0.002 μ g/ml pCMV-Rl (Promega) using 4 μ l of FuGENE6 reagent (Roche Molecular Biochemicals)/ μ g of DNA according to the manufacturer's protocol. The next day, cells were seeded in equal amounts in 24-well dishes and incubated for 16 h in low serum (0.5–2%) followed by treatment with 1 ng/ml TGF β 1 for 4 or 16 h. Firefly luciferase (Luc) and *Renilla reniformis* luciferase (RLuc) activities in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luc activity was normalized to RLuc activity and presented as relative luciferase units. All assays were done in triplicate wells, and each experiment was repeated at least twice.

Immunofluorescent Microscopy—NMuMG cells (10⁵ cells/well) were grown in DMEM, 5% FBS on glass coverslips (22 \times 22 mm) for 24 h before treatment with 2 ng/ml TGF β 1. Cells were fixed with methanol for 10 min at –20 °C or with 2% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. For permeabilization, cells were incubated with 0.1% Triton X-100 for 5 min at room temperature. Cells were washed three times in PBS after each treatment. Cells were blocked with 3% milk in PBS for 30 min at room temperature, incubated with primary antibodies diluted in 1% milk/PBS (1/300 for ZO-1, 1/500 for integrin β_1 , and 1/2000 for E-cadherin), and then incubated with fluorescent secondary antibodies (1/500) for 1 h at room temperature. Coverslips were mounted onto 25 \times 75-mm microslides (VWR Scientific, West Chester, PA) using AquaPolyMount (Polysciences, Warrington, PA). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

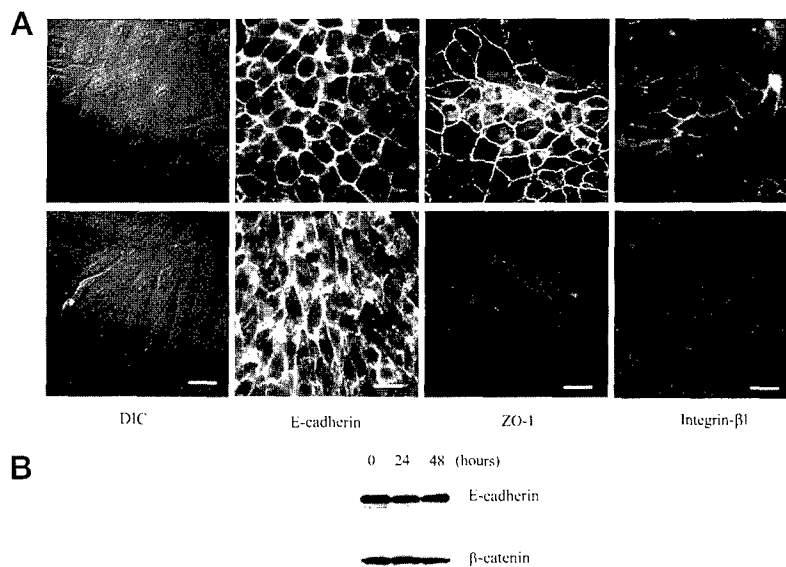
Migration Assays—4T1 and EMT6 tumor cells (4 \times 10⁴/well) were plated in DMEM, 10% FBS in the upper chamber of 8- μ m pore (24-well) transwells (Corning Costar, Cambridge, MA) and incubated alone or with variable concentrations of TGF β 1 in the absence or presence of LY294002 or the TGF β 1-neutralizing 2G7 IgG₂. Three days later, the cells that had migrated through pores and reattached to the lower chamber were trypsinized and cell numbers measured in a Coulter counter.

RESULTS

The PI3K-Akt Pathway Is Involved in EMT Induced by TGF β 1—TGF β 1 induced a mesenchymal transition in NMuMG cells within 24 h. Cells treated with 2 ng/ml TGF β 1 changed their shape from a cuboidal to a more elongated form (Fig. 1A, DIC). Concomitantly, TGF β 1 induced the delocalization of E-cadherin from adherens junctions, ZO-1 from tight junctions, and the delocalization of integrin β_1 from the cell surface (Fig. 1). There were no detectable differences in the intracellular staining of E-cadherin, ZO-1, and integrin β_1 between treated and untreated cells. In addition, no detectable changes in E-cadherin were found by immunoblot analysis of whole cell extract (Fig. 1B).

To determine the signaling pathways that contribute to TGF β -induced EMT, we examined the ability of different pharmacological agents to block the changes in cell morphology and in localization of epithelial markers at cell junctions. We found that LY294002, a synthetic inhibitor of the p110 catalytic subunit of PI3K (30), blocked the morphological transition, the delocalization of ZO-1 from cell junctions, and the reorganization of actin fibers (Fig. 2A). Inhibitors of MEK1/2 (PD098059 (Fig. 2A) and U0126), *c-jun* N-terminal kinase (curcumin), mTOR (mammalian target of rapamycin), phospholipase C (U73122), Rac1 (SCH51344), MLCK (myosin light chain kinase; ML7), and PP2A (okadaic acid) did not affect TGF β -mediated transition (data not shown), suggesting that signaling pathways associated with these molecules may not contribute to EMT mediated by TGF β 1. Inhibition of EMT by LY294002 suggested that PI3K is involved in EMT induced by

FIG. 1. TGF β 1-mediated EMT in NMuMG cells. A, NMuMG mammary epithelial cells were grown on glass coverslips for 24 h and treated (bottom row) or not (top row) with 2 ng/ml TGF β 1 for an additional 24 h. Differential interference contrast (DIC) images show changes in cell morphology in response to TGF β 1. Antibodies to ZO-1 (1:300), E-cadherin (1:2000), and integrin β_1 (1:500) were used to visualize cell junctions as indicated under "Experimental Procedures." Scale bars represent 15 μ m. B, immunoblot analysis of E-cadherin (1:2000) and α -catenin (1:2000) in protein extracts (50 μ g/lane) from control NMuMG cells or from cells treated with 2 ng/ml TGF β 1 for 24 or 48 h.



TGF β 1. To further test this hypothesis, NMuMG cells were infected with adenovirus encoding a constitutively active mutant of p110 (ca-p110), the catalytic subunit of PI3K. Cells expressing Myc-tagged ca-p110 showed a higher level phosphorylation of Akt at Ser-473, confirming its functional activity (Fig. 2B). Similar to exogenous TGF β 1, infection with the ca-p110 virus resulted in the delocalization of ZO-1 from tight junctions. However, the cells retained their epithelial morphology, whereas infection with a β -galactosidase adenovirus (Ax β -Gal) did not alter cell morphology nor ZO-1 staining at adherens junctions (Fig. 2B). Finally, we examined whether Akt/PKB, a downstream effector of PI3K, would affect EMT. Transduction of NMuMG cells using a dominant-negative mutant Akt (AktK179D) adenovirus inhibited TGF β -induced delocalization of ZO-1 from tight junctions as well as changes in cell morphology (Fig. 2C). These data suggest that the PI3K-Akt pathway is required for some of the phenotypic hallmarks associated with TGF β -mediated EMT.

Activation of the PI3K-Akt Pathway in Response to TGF β 1—To further test that the PI3K pathway is activated by TGF β 1, we examined the phosphorylation status and kinase activity of Akt. Immunoblot analyses with antibodies specific to the phosphorylated form of Akt showed that TGF β induced phosphorylation of Akt at Ser-473 within 30 min, achieving a detectable maximum at 2 h (Fig. 3A). Phosphorylation of Ser-473 Akt was inhibited by 20 μ M LY294002 (Fig. 3A, last lane), indicating that Akt activation requires PI3K function. The activity of Akt/PKB was measured using an *in vitro* kinase assay with GST-GSK3 β fusion protein containing GSK-3 β peptide in frame with GST and immobilized on agarose beads as a substrate. Treatment of NMuMG cells with TGF β 1 for 2 h stimulated a 4-fold induction in the incorporation of 32 P into GST-GSK3 β (Fig. 3B). Next, we tested the TGF β 1 dose dependence of phosphorylation of Akt and Smad2. Treatment with 0.5 ng/ml (20 pM) TGF β 1 was sufficient to induce a maximal phosphorylation for both Ser-473 Akt and Smad2 (Fig. 3C). TGF β 1 and EGF, a known agonist of PI3K, induced similar levels of Ser-473 Akt phosphorylation. EGF induced activating phosphorylation of ERK1/2, whereas TGF β 1 did not stimulate ERK activation at any concentration tested (Fig. 3C).

Rho-like GTPases Mediate Activation of the PI3K-Akt Pathway in Response to TGF β 1—Recent studies have suggested that RhoA is involved in TGF β 1-mediated transcription (22,

23) and that TGF β 1 can activate RhoA in NMuMG cells.² Therefore, we tested whether RhoA GTPase affected the activation of PI3K-Akt mediated by TGF β 1. NMuMG cells transiently transfected with a dominant-negative RhoA mutant (N19RhoA) showed a significantly reduced level of Akt phosphorylation compared with a control (Fig. 3D). Transfection of the constitutively active form of RhoA (Q61LRhoA) resulted in an increase of basal phosphorylation of Akt (Fig. 3E). These results suggest that RhoA may be involved in TGF β 1-mediated activation of the PI3K-Akt pathway.

Transcriptional Responses to TGF β 1 Involve the PI3K-Akt Pathway—TGF β transcriptional responses can be controlled through the subcellular localization of Smads. It has been shown that SARA, a recently identified mediator of TGF β signaling, controls recruitment of Smad2 to TGF β receptors (31). The function of SARA depends on its FYVE homology domain, which binds phosphatidylinositols phosphorylated by PI3K (31). In addition, recent data have suggested that microtubules (MTs) may control Smad-dependent TGF β 1 transcriptional responses (32). It has been shown that PI3K associates tightly with α - and β -tubulins (33), and it is involved in the function of MTs (34). Therefore, we next examined whether PI3K is involved in the regulation of TGF β -mediated transcription. Two TGF β -responsive reporter constructs were used in transcriptional assays: p3TP-Lux, containing the firefly luciferase reporter gene under the control of three 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements and a fragment of the PAI-1 promoter (1), and p(CAGA)₁₂-Lux, a reporter gene containing 12 repeats of Smad binding sequences from the PAI-1 promoter (35). In NMuMG cells transiently transfected with p3TP-Lux, TGF β -mediated induction of luciferase was inhibited by LY294002 in a dose-dependent manner at 4 and 16 h (Fig. 4A). Similar results were obtained with 4T1 and EMT6 mammary tumor cell lines (data not shown). LY294002 also inhibited TGF β -stimulated reporter activity in both NMuMG and 4T1 cells transfected with p(CAGA)₁₂-Lux (Fig. 4B). We next examined whether an adenovirus vector encoding a dominant-negative mutant of p85 (dn-p85), the regulatory subunit of PI3K, would emulate the effects of LY294002. Expression of dn-p85 significantly reduced a basal phosphoryla-

² N. A. Bhowmick, M. Ghiassi, A. V. Bakin, M. Aakre, C. A. Lundquist, M. E. Engel, C. L. Arteaga, and H. L. Moses, submitted for publication.

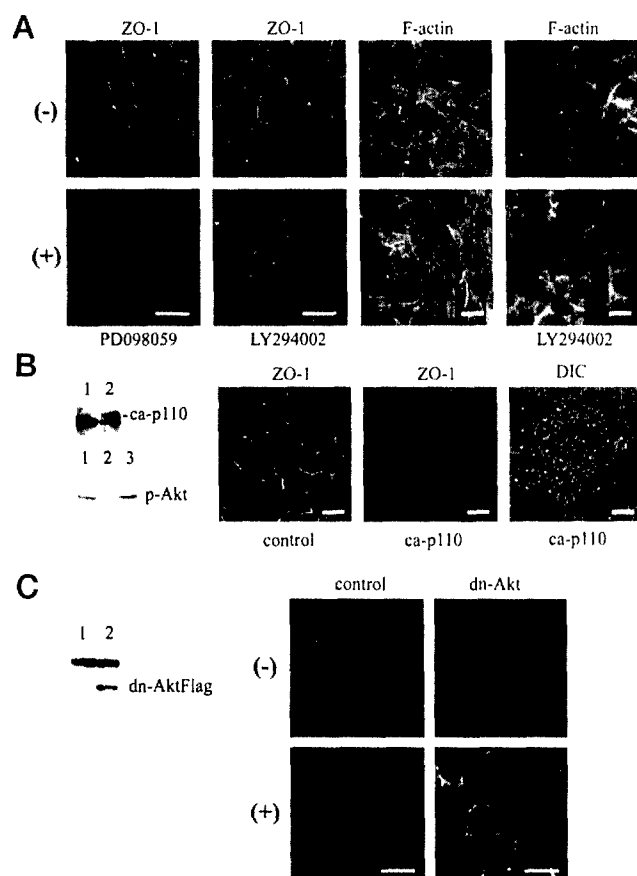


FIG. 2. The PI3K-Akt pathway is involved in TGF β -induced EMT. **A**, NMuMG cells were treated or not with 2 ng/ml TGF β 1 for 24 h in the presence 20 μ M PD098059 or 20 μ M LY294002 where indicated. Cells were stained with antibodies to ZO-1 (1:300) or phalloidin-fluorescein isothiocyanate (1:100) to visualize actin filaments. **B**, localization of ZO-1 in NMuMG cells infected with adenovirus vectors encoding β -galactosidase or the constitutively active mutant of p110 (ca-p110) for 48 h at a multiplicity of infection of 100 plaque-forming units/cell. By phase contrast (DIC), differential interference contrast, cells infected with the ca-p110 virus retained their epithelial morphology. The immunoblot analysis shows expression of Myc-tagged ca-p110 (lane 2) in cells infected with ca-p110 compared with control virus (lane 1). The lower panel shows the level of phospho-Ser-473 Akt in control cells (lane 1), cells infected with dn-p85 (lane 2), or cells infected with ca-p110 (lane 3). **C**, NMuMG cells were infected with AxAkt-K179D (dn-Akt) or Ax β -Gal (control) adenoviruses at a multiplicity of infection of 40; 48 h later, cells were treated with 2 ng/ml TGF β 1 for an additional 24 h followed by immunostaining for ZO-1 (1:300) as indicated under "Experimental Procedures." The immunoblot shows expression of Flag-tagged dn-Akt in cells infected with AxAkt-K179D (lane 2) compared with the control virus (lane 1). Scale bars represent 15 μ m.

tion of Ser-473 Akt (Fig. 2B), confirming its functional activity. TGF β -induced p3TP-Lux reporter activity was reduced by 75% in both NMuMG and 4T1 cells infected with the dn-p85 adenovirus vector but not with a control adenovirus encoding β -galactosidase (Fig. 4C). Finally, transient transfection of a dominant-negative mutant of Akt (AktK179M) markedly inhibited TGF β -induced p3TP-Lux transcription (Fig. 4D). These data suggest that the PI3K-Akt pathway is involved in TGF β transcriptional responses.

TGF β 1-mediated Phosphorylation of Smad2 Requires PI3K—The transcriptional data using the p(CAGA)₁₂Lux reporter (Fig. 4B) suggested that PI3K is involved in the control of Smad-dependent transcription. Therefore, we examined the effect of PI3K blockade on TGF β -induced phosphorylation of Smad2. Immunoblot analysis with antibodies specific to Smad2 phosphorylated at the C terminus showed that C terminus phosphorylation of Smad2 was induced by TGF β 1 within 15

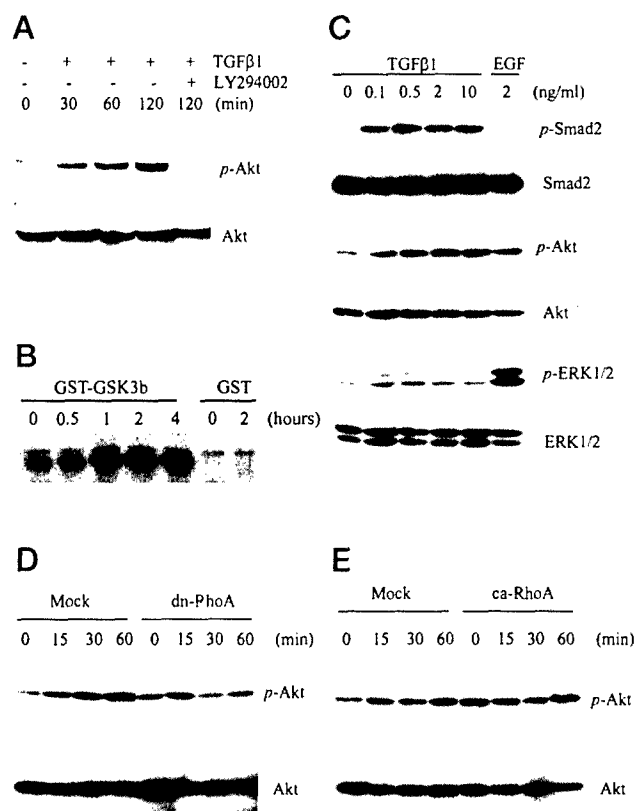
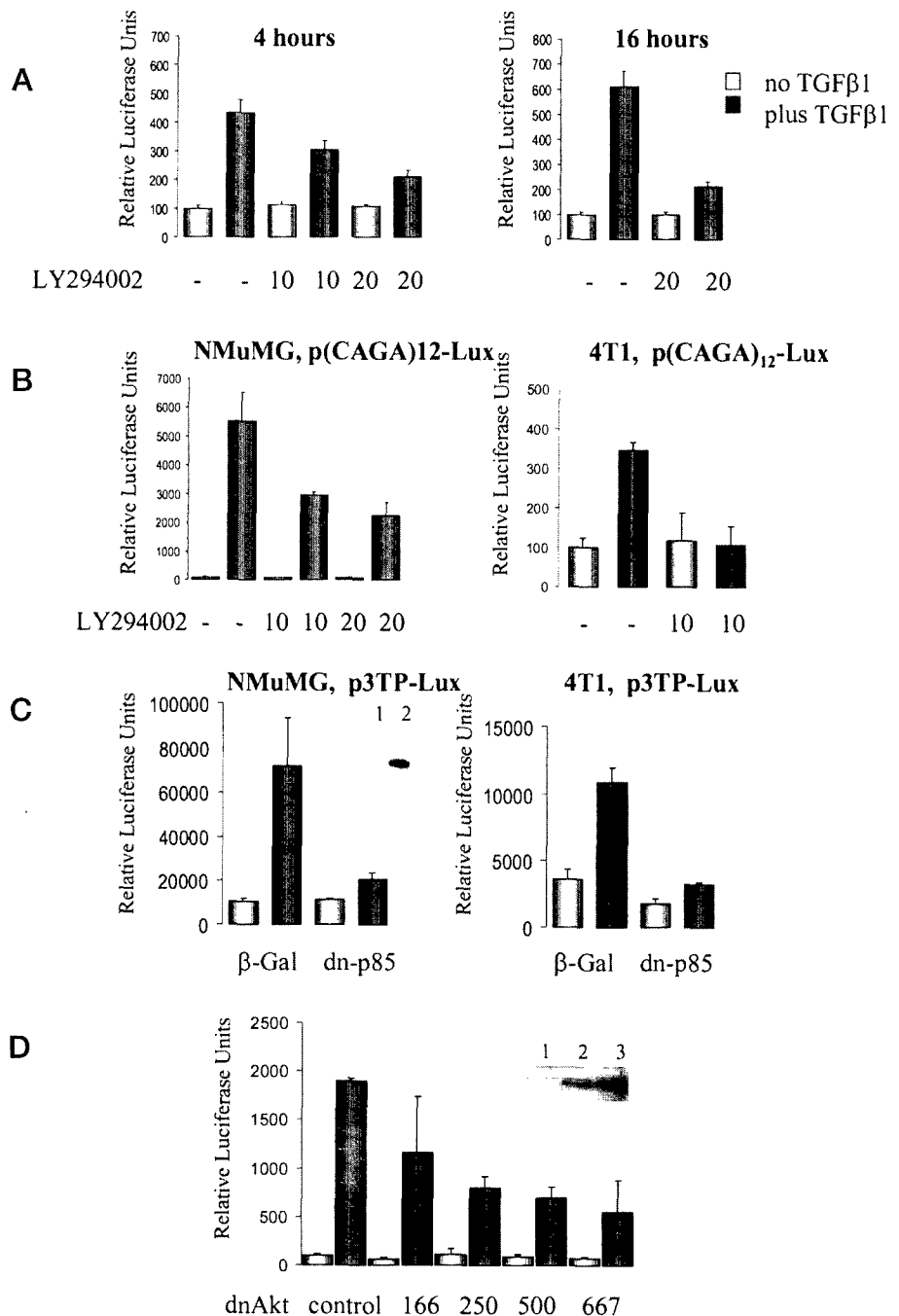


FIG. 3. TGF β 1 activates the Akt/PKB kinase in NMuMG cells. **A**, NMuMG cells were stimulated with 2 ng/ml TGF β 1 for the indicated times. After lysis in EBC buffer, protein extracts (50 μ g/lane) were subjected to SDS-PAGE followed by immunoblot analysis with antibodies for phospho-Ser-473 Akt (1:1000) or total Akt (1:1000). **B**, Akt/PKB kinase activity in protein extracts (150 μ g) from NMuMG cells treated with 2 ng/ml TGF β 1 was determined by an *in vitro* kinase assay as described under "Experimental Procedures." Phosphorylated GST-GSK-3 β was resolved by SDS-PAGE, and ³²P incorporation was analyzed by PhosphorImager. **C**, immunoblot analysis of protein extracts from NMuMG cells treated with different concentration of TGF β 1 or 2 ng/ml EGF for 60 min using phospho-specific antibodies to phospho-Smad2, Akt, and ERK1/2. **D**, immunoblot analysis of phospho-Ser-473 Akt and total Akt in cells transiently transfected with a dominant-negative RhoA mutant (dn-RhoA). **E**, immunoblot analysis of phospho-Ser-473 Akt and total Akt in cells transiently transfected with a constitutively active RhoA mutant (ca-RhoA).

min, reaching a maximum by 1 h. However, co-incubation with 20 μ M LY294002 markedly reduced ligand-mediated Smad2 phosphorylation without detectable changes in total Smad2 protein levels (Fig. 5A). At the same time, phosphorylation of Ser-473 Akt was completely blocked by LY294002 (Fig. 5B). The induction of the C-terminal phosphorylation of Smad2 and phosphorylation of Ser-473 Akt in response to TGF β 1 appears to occur with similar kinetics and TGF β 1 dose dependence (Figs. 3 and 5). To test whether the PI3K-Akt pathway is directly involved in the C-terminal phosphorylation of Smad2, NMuMG cells were transfected with dn-Akt followed by TGF β 1 treatment and immunoblot analysis of C terminus phosphorylation of Smad2. The level of Smad2 phosphorylation was similar in control cells and cells transfected with dn-Akt, suggesting that Akt is not involved in C-terminal phosphorylation of Smad2 (Fig. 5C). Infection of cells with ca-p110 also did not induce ligand-independent phosphorylation of Smad2 (Fig. 5D).

TGF β 1-induced Cell Migration Requires PI3K Activity—TGF β 1 can stimulate the migration of tumor and nontumor cells (7, 36, 37). PI3K has been implicated in the regulation of cell migration and chemotaxis of human neutrophils (38–40).

FIG. 4. Blockade of PI3K-Akt abrogates transcriptional responses to TGF β 1. A, NMuMG were transfected with p3TP-Lux and pCMV-Rl vectors, starved for 16 h in 1% FBS, and stimulated with 1 ng/ml TGF β 1. Cells were lysed 4 or 16 h thereafter and assayed for dual-luciferase activity as described under "Experimental Procedures." Relative luciferase units represent the ratio of firefly to *Renilla* luciferase activities. Each data point represents the mean \pm S.D. of 3 wells. B, relative luciferase units in NMuMG and 4T1 cells transfected with p(CAGA)₁₂-Lux and pCMV-Rl vectors and treated with 1 ng/ml TGF β 1 for 16 h in the absence or presence of LY294002. Each bar represents the mean \pm S.D. of 3 wells. C, analysis of luciferase activity in NMuMG or 4T1 cells transduced with an adenoviral vector encoding a dominant-negative mutant of p85 (dn-p85) or β -galactosidase (β -gal) and subsequently transfected with the p3TP-Lux vector. Luciferase activity was measured as indicated under "Experimental Procedures" and normalized to the protein concentration. Each data point represents the mean \pm S.D. of 6 wells. Immunoblot analysis shows expression of dn-p85 in cells infected with a control virus (first lane) or with dn-p85 virus (second lane). D, NMuMG cells were transfected with reporter vectors and the indicated amounts of plasmid encoding AktK179M, a dominant-negative Akt mutant (dn-Akt), and/or pcDNA3 empty vector (control) for a combined total of 667 ng of ectopic plasmid DNA. After treatment with 1 ng/ml TGF β 1 or no treatment for 16 h, the relative luciferase units from triplicate wells were measured as described under "Experimental Procedures." The immunoblot detects HA-tagged dn-Akt in cells transfected with a control plasmid or with 250 ng (lane 2) or 667 ng (lane 3) of dn-Akt.



Therefore, we examined whether PI3K is involved in TGF β -induced cell migration. We used 4T1 and EMT6 mouse tumor cells, which exhibit high levels of TGF β receptors that mediate transcriptional responses (Fig. 4) but are not growth inhibited by exogenous TGF β 1.³ TGF β 1 enhanced migration of both cell lines in a dose-dependent manner with an EC₅₀ of approximately 0.1 ng/ml (4 pM). LY294002 blocked both basal and TGF β -stimulated cell migration (Fig. 6A) without an effect on tumor cell proliferation (data not shown). The TGF β 1-neutralizing 2G7 monoclonal antibody also reduced basal cell migration, suggesting that this phenotypic response was partially dependent on autocrine TGF β signaling (Fig. 6B). Furthermore, both LY294002 and 2G7 reduced the basal level of phosphorylation at Ser-473 Akt in 4T1 and EMT6 cells (Fig. 6C), suggesting a causal association between autocrine TGF β sig-

naling with basal PI3K-Akt signaling and the subsequent migration of tumor cells.

DISCUSSION

The tumor-promoting activity of TGF β 1 associated with the induction of EMT has been documented for different tumor types (5–9). Several reports have shown that TGF β can induce a reversible mesenchymal transition in mammary epithelial NMuMG cells (11, 12). In this study, we present data to support the role of the PI3K-Akt pathway in TGF β -mediated EMT. We found that either the blockade of PI3K activity by a synthetic inhibitor, LY294002, or by expression of dn-Akt significantly inhibited EMT (Fig. 2). These observations led us to hypothesize that the PI3K-Akt pathway is directly involved in this transition. Similar to TGF β 1, forced expression of constitutively active PI3K (ca-p110) was sufficient to promote the disruption of cellular junctions but did not induce *per se* the

³ C. L. Arteaga, unpublished data.

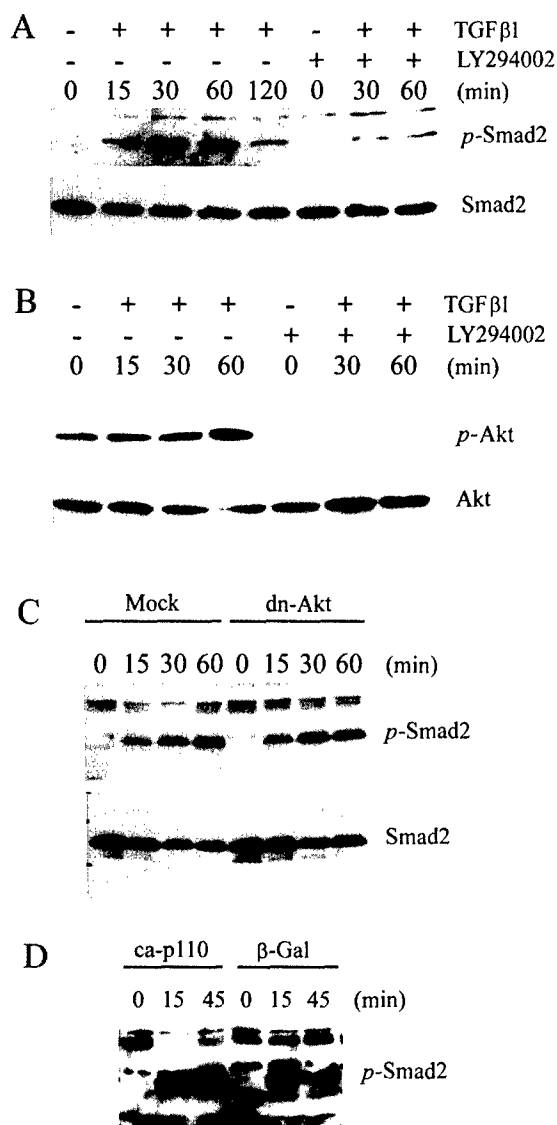


FIG. 5. Blockade of PI3K inhibits TGF β -mediated C-terminal phosphorylation of Smad2. NMuMG cells were treated with 2 ng/ml TGF β 1 for the indicated times in the presence or absence of 20 μ M LY294002. Protein extracts (50 μ g/lane) were separated by 12.5% SDS-PAGE followed by immunoblot analysis for phospho-Smad2 (1:500) and total Smad2 (1:500) (A) or phospho-Ser-473 Akt (1:1000) and total Akt (1:1000) (B), as indicated under "Experimental Procedures." C, immunoblot analysis of phospho-Smad2 and total Smad2 in cells transiently transfected with AktK179M, a dn-Akt mutant. D, immunoblot analysis of phospho-Smad2 in cells infected with a ca-p110. β -Gal, β -galactosidase.

changes in cell morphology associated with EMT (Fig. 2B). The dissolution of tight junctions and the disruption of adherent junctions induced by TGF β 1 are relatively early processes, occurring within 4–8 h after the addition of TGF β 1, whereas changes in the cell shape occur later. This result suggests that PI3K function is required for the early changes during TGF β -mediated EMT but that other events associated with the reorganization of cytoskeleton leading to changes in cell morphology may not depend on the PI3K-Akt pathway. The observed delocalization of E-cadherin, integrin β 1, and ZO-1 from cellular junctions occurred without detectable changes in their cellular content, suggesting that these TGF β -mediated effects may involve PI3K-dependent endocytosis. These observations are consistent with the studies implicating PI3K in endocytosis and vesicular trafficking (41–43). Similar to TGF β , hepatocyte growth factor can also disrupt epithelial cell-cell junctions and

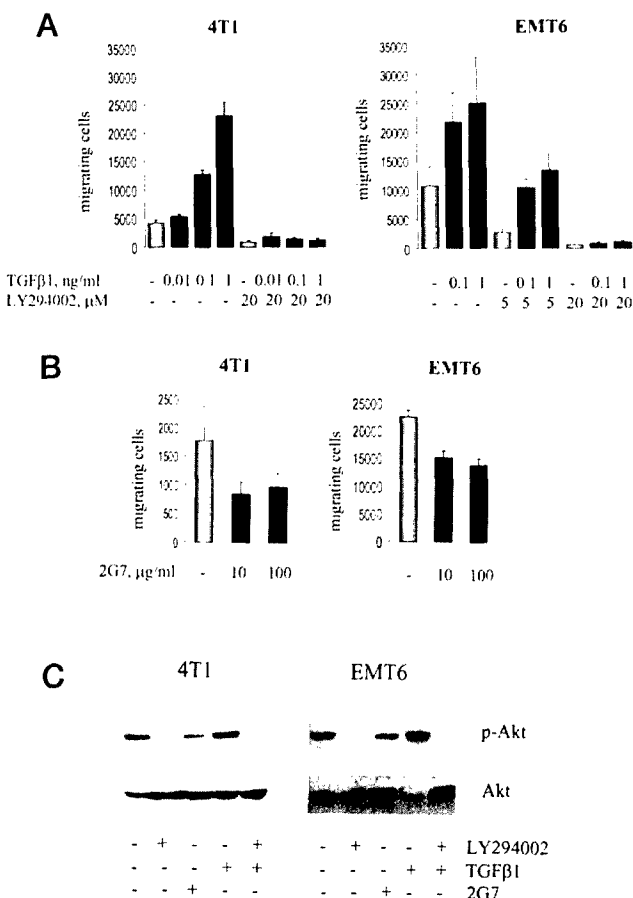


FIG. 6. Basal and TGF β -stimulated tumor cell migration and Akt kinase activity are reduced by LY294002. A, 4T1 and EMT6 tumor cells (4×10^4 cells/well) were seeded in the upper chamber of 8- μ m pore transwells and incubated with TGF β 1 in the absence or presence of LY294002. Cells that migrated through the polycarbonate filters and attached to the bottom chamber were counted 3 days later. Each bar represents the mean \pm S.D. of 3 wells. B, 4T1 and EMT6 cells were seeded under identical conditions as described in A in the absence or presence of the TGF β -neutralizing monoclonal antibody 2G7. Cells migrating through the 8- μ m pores were counted 3 days later. Data represent the mean \pm S.D. of 3 wells. C, exponentially growing 4T1 and EMT6 cells in DMEM, 5% FBS were incubated with 1 ng/ml TGF β 1 with or without 20 μ M LY294002 for 4 h. Where indicated, the 2G7 monoclonal antibody (10 μ g/ml) was added for 24 h. Whole cell lysates were prepared, and 50 μ g of total protein/lane were subjected to SDS-PAGE followed by immunoblot analyses for phospho-Ser-473 Akt and total Akt as indicated under "Experimental Procedures."

induce the delocalization of E-cadherin from cell junctions (44). In this process, hepatocyte growth factor induces the delocalization of both E-cadherin and the hepatocyte growth factor receptor, c-Met, via PI3K-mediated co-endocytosis (44). This co-endocytosis can be blocked by dominant-negative mutants of RhoA and Rab5, a component of early endosomes (44). In addition, Rab5-mediated endocytosis is also regulated by Akt/PKB (45). Thus, TGF β -mediated delocalization of epithelial markers from cell junctions may involve the function of PI3K-Akt and Rho-like GTPases.

The activation of PI3K in response to TGF β has been reported in two other cell systems (24, 25). In NMuMG cells, TGF β 1 induced phosphorylation and activation of Akt/PKB with kinetics similar to the C-terminal phosphorylation of Smad2 (Figs. 3 and 5). Activation of Akt depends on PI3K, since it can be blocked by a synthetic inhibitor of PI3K (Figs. 3 and 5) and by expression of dn-p85 (Fig. 2B, inset). These results suggest that the PI3K-Akt pathway is activated directly by TGF β 1. This conclusion is further supported by recent reports showing co-precipitation of p85, the regulatory subunit of

PI3K, with TGF β receptors and stimulation of PI3K activity by TGF β 1 in other cell types (24, 25). We also confirmed a direct association p85 with both type I and type II TGF β receptors in NMuMG cells.⁴

Because of the reported role of Rho family GTPases in TGF β 1 signaling and their interaction with the PI3K pathway (46), we tested the role of the RhoA GTPase in TGF β -mediated activation of Akt. Expression of dominant-negative N19RhoA mutant disrupted ligand-induced phosphorylation of Akt at Ser-473. On the other hand, expression of a constitutively active mutant, Q63LRhoA, resulted in an increase of the basal phosphorylation of Akt. These findings suggest that RhoA GTPase is involved in TGF β 1-mediated activation of Akt, which is consistent with recent reports that Rho-like GTPases can synergize with TGF β signaling (22, 23). Therefore, RhoA may function as an upstream effector of Akt activation in response to TGF β 1.

Using two reporter constructs, p3TP-Lux and p(CAGA)₁₂-Lux, we found that TGF β 1 transcriptional responses in NMuMG and two tumor cell lines are inhibited by both pharmacological and molecular antagonists of the PI3K-Akt pathway, including dominant-negative p85 and Akt mutants (Fig. 4, A-D). The fact that a blockade of the PI3K-Akt pathway affected Smad-dependent transcriptional responses suggested the involvement of PI3K and Akt in TGF β intracellular signal transduction. Consistent with this idea, we found that LY294002 significantly reduced TGF β -mediated C-terminal phosphorylation of Smad2 in NMuMG cells (Fig. 5). However, neither PI3K nor Akt is involved in C-terminal phosphorylation of Smad2, since introduction of ca-p110 or dn-Akt did not affect it. These results, coupled with the inhibitory effect of LY294002 on Smad2 phosphorylation (Fig. 5), suggest that PI3K is involved indirectly in TGF β -mediated C-terminal phosphorylation of Smad2.

PI3K activity may also be required for the function of intracellular mediators of TGF β signaling. Recently, two factors regulating C-terminal phosphorylation of Smad2 were described (31, 32). First, the intracellular localization of Smad2 is controlled by SARA, a recently cloned Smad2-binding protein (31). SARA co-localizes with EEA1, an early endosome marker,⁵ and this co-localization depends on the FYVE domain of SARA, which binds phosphatidylinositol 3-phosphates (47, 48). It has been shown that deletion of the FYVE domain results in the mislocalization of Smad2 and inhibition of TGF β transcriptional responses (31). We found that Smad2 co-localizes with EEA1 in the absence of TGF β in NMuMG cells.⁶ Thus, it is conceivable that the blockade of PI3K activity in NMuMG cells with LY294002, similar to wortmannin (49), will reduce the levels of phosphatidylinositol 3-phosphate, resulting in the mislocalization of Smad2. This is a potential explanation of the inhibitory effect of LY294002 on TGF β -induced phosphorylation of Smad2 (Fig. 5A), whereas neither ca-p110 nor dn-Akt can directly modulate Smad2 phosphorylation (Fig. 5C, D). In addition, a recent report provides evidence that endogenous Smad2, Smad3, and Smad4 are stored in the MT network (32). It has been suggested that upon TGF β treatment, Smad2 and Smad3 dissociate from MT, become phosphorylated by T β RI, and translocate to the nucleus where they regulate the transcription of TGF β target genes. Moreover, destabilization of MTs with nocodazole can facilitate Smad-mediated TGF β transcriptional responses *per se* in the absence of exogenous TGF β 1 (32). On the other hand, TGF β has been reported to stabilize MTs (50), potentially limiting Smad signaling. PI3K has also

been shown to control the dynamics of the MT network, which is important for intracellular trafficking, cell motility, and other cell functions (51). Therefore, PI3K antagonists may affect the MT network and interfere with TGF β signaling. To formally demonstrate that PI3K blockade inhibits TGF β signaling via its effects on MTs will require further investigation.

Both TGF β and PI3K have been implicated in chemotaxis and cell migration (7, 36-40). Here, we show that pm concentrations of TGF β 1 enhanced the basal migration of tumor cells, whereas blockade of PI3K with LY294002 reduced both basal and TGF β -stimulated cell migration (Fig. 6, A and B). These data are in agreement with a critical role of PI3K in cell motility and migration via the modulation of cytoskeletal organization (47, 51). These results were generated with tumor cells that exhibit high levels of TGF β expression and TGF β receptors as well as constitutive activation of Akt in the absence of added TGF β ligand. Similar to LY294002, TGF β 1-neutralizing monoclonal antibodies reduced basal cell migration and Ser-473 phosphorylation of Akt, suggesting an association between autocrine TGF β signaling with both constitutively activated Akt/PKB and cell invasiveness. Neither exogenous TGF β , anti-TGF β antibodies, nor LY294002 had any effect on 4T1 or EMT6 cell proliferation. These data coupled with the transcription data using TGF β reporters in 4T1 and EMT6 cells imply that EMT can be dissociated from the anti-mitogenic effects of TGF β . In summary, the results presented provide evidence that the PI3K-Akt pathway is causally involved in the morphogenic, transcriptional, and migratory activities of TGF β .

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p38 mitogen-activated protein kinase is required for TGF β -mediated fibroblastic transdifferentiation and cell migration

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RUNNING TITLE

p38Mapk and TGF β -mediated EMT

SUMMARY

Transforming growth factor β (TGF β) contributes to tumor progression by inducing an epithelial to mesenchymal transdifferentiation (EMT) and cell migration. We found that TGF β -induced EMT was blocked by inhibiting activation of p38Mapk with H-7, a protein kinase inhibitor, or p38Mapk activity with SB202190, a p38Mapk inhibitor, suggesting involvement of p38Mapk in EMT. Inhibition of the p38Mapk pathway affected TGF β -mediated phosphorylation of ATF2, but did not inhibit phosphorylation of Smad2. SB202190 impaired TGF β -mediated changes in cell shape and reorganization of the actin cytoskeleton. Forced expression of dominant negative (DN) MKK3 inhibited TGF β -mediated activation of p38Mapk and EMT. Expression of DN-p38 α impaired TGF β -induced EMT. Inhibition of p38Mapk blocked TGF β -induced migration of non-tumor and tumor mammary epithelial cells. TGF β induced activation of the p38Mapk pathway within 15 min. Expression of TGF β type II (T β RII) and type I (T β RI/Alk5) kinase-inactive receptors blocked EMT and activation of p38Mapk, whereas expression of constitutively active Alk5-T204D resulted in EMT and phosphorylation of MKK3/6 and p38Mapk. Finally, dominant negative Rac1N17 blocked TGF β -induced activation of the p38Mapk pathway and EMT, suggesting that Rac1 mediates activation of the p38Mapk pathway. These studies suggest that the p38Mapk pathway is required for TGF β -mediated EMT and cell migration.

INTRODUCTION

The transforming growth factor β (TGF β) family of secreted factors regulates various biological processes, including cell proliferation, differentiation, and apoptosis (Massague, 1998). TGF β s signal through cell-surface serine-threonine kinase type II and type I receptors. TGF β binding to TGF β type II (T β RII) receptor triggers its association with the TGF β type I (T β RI) receptor (Massague, 1998). T β RII phosphorylates and activates T β RI, which, in turn, phosphorylates receptor-associated (RA) Smads (Smad2 and Smad3). RA-Smads bind Smad4 and translocate to the nucleus where they regulate transcription of target genes (Massague, 1998). In addition to Smads, TGF β can activate Jun N-terminal kinase (JNK) (Atfi et al., 1997; Engel et al., 1999; Frey and Mulder, 1997), extracellular signal-regulated kinase (ERK) (Hartsough and Mulder, 1995), p38 mitogen activating protein kinase (p38Mapk) (Hanafusa et al., 1999), and Akt (Bakin et al., 2000).

Smad-dependent signaling has been shown to be required for the antiproliferative activity of TGF β , and components of this pathway are frequently mutated or silenced in several human cancers (de Caestecker et al., 2000). Tumors, however, frequently express high levels of TGF β and inhibition of TGF β signaling has been shown to reduce tumor invasiveness and metastasis (Akhurst and Balmain, 1999; Barrack, 1997; Cui et al., 1996; Hojo et al., 1999). A number of studies provide evidence that TGF β contributes to tumor cell invasion and metastasis by inducing mesenchymal transdifferentiation in epithelial cells (EMT) and stimulating cell migration (Akhurst and Balmain, 1999; Barrack, 1997; Oft et al., 1998). This TGF β -mediated fibroblastic transdifferentiation is a complex process associated with alterations in epithelial cell junctions, changes in cell morphology, reorganization of the cell cytoskeleton, expression of fibroblastic markers (fibronectin, vimentin), and enhancement of cell migration (Bakin et al., 2000; Miettinen et al., 1994; Piek et al., 1999b).

The molecular mechanisms of TGF β -mediated EMT and cell migration are not entirely understood. Studies with TGF β receptors have shown that a truncated TGF β /BMP type I receptor, Alk2, blocks EMT in mouse NMuMG cells (Miettinen et al., 1994). Adenoviral expression of constitutively active human T β RI/Alk5 together with Smad2/3 can induce EMT in these cells (Piek et al., 1999b). Expression of a dominant negative truncated form of T β RII decreases the formation of invasive spindle tumours (Portella et al., 1998). Adenoviral expression of Smad2/3 induced EMT only in the context of expression of constitutively active Alk5 (Piek et al., 1999b). Overexpression of Smad7, an inhibitor of Smad-dependent signaling, or dominant negative Smad3 did not affect the transdifferentiation, arguing against involvement of Smads in EMT (Bhowmick et al., 2001a). Inhibition of JNK with curcumin (Bakin et al., 2000) or by expression of dominant negative JNK mutant (Bhowmick et al., 2001a) did not affect EMT. TGF β did not activate the Ras-Raf-ERK1/2 cascade and MEK inhibitors (PD098059 and U0126) did not block EMT in NMuMG cells (Bakin et al., 2000; Piek et al., 1999b). We have recently shown that the PI3K-Akt pathway contributes to EMT at the step of tight junctions disruption (Bakin et al., 2000). The role of p38Mapk in TGF β -mediated EMT has not been studied.

The p38Mapk pathway has been implicated in various biological responses to members of the TGF β superfamily including TGF β -stimulated migration of smooth muscle cell (Hedges et al., 1999), neuronal differentiation of PC12 cells induced by bone morphogenic protein-2 (BMP-2) (Iwasaki et al., 1999), growth/differentiation factor-5 induced chondrogenesis of ATDC-5 cells (Nakamura et al., 1999), and BMP-mediated cardiomyocyte differentiation (Monzen et al., 1999). Studies in *Drosophila* have shown that p38Mapks are required for wing morphogenesis downstream of decapentaplegic (Dpp), a homologue of TGF β (Adachi-Yamada et al., 1999). The p38Mapk

pathway has also been implicated in TGF β transcriptional responses (Hanafusa et al., 1999; Kucich et al., 2000; Sano et al., 1999).

The molecular mechanism(s) of TGF β -induced activation of p38Mapk signaling are not defined. Mammalian p38Mapks are activated by distinct upstream dual specificity MAP kinase kinases (MKK), MKK3 and MKK6 (Tibbles and Woodgett, 1999). TGF β activated kinase-1 (Tak1) phosphorylates MKK3/6 in TGF β and BMP signaling (Shibuya et al., 1998; Yamaguchi et al., 1995). In addition, other MKK kinases including p21-activating kinase (PAK1) and mixed-lineage kinase (MLK) have been shown to phosphorylate Mapk kinases (MKK3/6) and induce p38Mapks (Tibbles et al., 1996; Zhang et al., 1995). p38Mapk downstream targets include MAPK activated protein kinase-2, mitogen- and stress-activated protein kinase-1 (MSK1), and transcription factors ATF2, CHOP, CREB, and MEF2C (Tibbles and Woodgett, 1999). Recent studies have found that p38Mapks are involved in the control of cell cytoskeleton and cell migration via phosphorylation of paxillin and heat shock protein HSP27 (Hedges et al., 1999).

In these studies we found that H-7, a protein kinase inhibitor, blocks TGF β -induced EMT and activation of the p38Mapk pathway in NMuMG mouse mammary epithelial cells. The specific p38Mapk inhibitors, SB203580 and SB202190, impaired TGF β -mediated changes in cell shape, the actin cytoskeleton, and cell migration. H-7 and the p38Mapk inhibitors blocked phosphorylation of ATF2, but did not inhibit TGF β -mediated phosphorylation of Smad2. Expression of dominant negative mutants (DN) of MKK3 or p38 α inhibited TGF β -mediated EMT. We also showed that TGF β activates the MKK3/6-p38Mapk-ATF2 cascade within 15 min and expression of DN-MKK3 blocked TGF β -mediated activation of p38Mapk and EMT. Kinase-inactive TGF β type II and type I (Alk5) receptors blocked EMT and the activation of p38Mapk. Forced expression of kinase-active Alk5-T204D induced both EMT and phosphorylation of p38Mapk in NMuMG cells. Alk5-T204D-

induced EMT was blocked by a p38Mapk inhibitor. Finally, we demonstrated that forced expression of dominant negative Rac1N17 blocked TGF β -induced activation of the p38Mapk-ATF2 cascade and EMT.

EXPERIMENTAL PROCEDURES

Antibodies and other reagents. TGF β 1 and TNF α were obtained from R&D Systems. Antibodies to fibronectin, Rac1 and Smad2 were from Transduction Laboratories; to ZO-1 from Chemicon; the monoclonal antibody to p38Mapk and rabbit polyclonal to haemagglutinin (HA) epitope were from Santa Cruz Biotechnology, Inc. Phalloidin-FITC, phalloidin-Texas Red, and Hoechst 3342 were from Molecular Probes. The β -tubulin-Cy3 antibody was from Sigma. Antibodies to phospho-Ser473 Akt, total Akt, phospho-MKK3/6, phospho-p38Mapk, and phospho-ATF2 were from New England BioLabs, and to C-terminal phospho-Smad2 from Upstate Biotechnology, Inc. LY294002, H-7, SB203580, and SB202190 were from Calbiochem. The GST-ATF2 fusion protein was from New England Biolabs. TBS buffer contained 20 mM Tris-HCl, pH 7.6, 150 mM NaCl. TBST was TBS supplemented with 0.1% Tween 20 (v/v).

Cell culture. NMuMG mouse mammary epithelial cells, SiHa human cervical carcinoma cells, MDA-MB-231 human breast cancer cells and HEK293T human kidney cells were purchased from American Tissue Culture Collection (ATCC). Cells were cultured as recommended by ATCC. 4T1 tumor cells were provided by F. Miller (Karmanos Cancer Center, Detroit, MI) and cultured in 10% FBS-DMEM.

Plasmids and Retroviral constructs. The retroviral vectors pGabe and pGabe-T β RII-K277R were provided by Martin Oft (UCSF, San Francisco, CA) and have been described previously (Oft et al., 1998). The T β RII-K277R construct contains an HA-tag at the N-terminus. Human wild type Alk5, dominant negative Alk5-K232R, and constitutively active Alk5-T204D constructs were provided by Masahiro Kawabata (The Cancer Institute, Tokyo, Japan). To generate pBMN-Alk5 constructs, the *EcoRI/SalI* fragments of Alk5 and Alk5-K232R including the C-terminal HA-tag were cloned in the retroviral pBMN-IRES-EGFP vector provided by Garry Nolan (Stanford University). The pBMN-Rac1N17 was engineered by cloning a *BamHI-XhoI* fragment encoding Rac1N17 from pCDNA3-Rac1N17 (a gift of Richard Cerione, Cornell University, Ithaca, NY) at the *BamHI-SalI* site of the retroviral pBMN-IRES-GFP vector. RhoAN19 and RhoAQ63L were previously described (Bakin et al., 2000). The pBMN-MKK3AL and pBMN-MKK6AL plasmids were generated by cloning *Sall-NotI* fragment of MKK6AL or *XhoI-NotI* fragment of MKK3AL from pCDNA3 vector into the retroviral pBMN-IRES-GFP vector. pCDNA3-MKK3AL and pCDNA3-MKK6AL plasmids were a gift of James Woodgett (The Ontario Cancer Institute, Toronto, Ontario). pBMN-p38AGF encoding a dominant negative mutant of p38 α and containing N-terminal Flag epitope was generated by cloning *HindIII-XbaI* fragment of p38AGF from pcDNA3-p38AGF at *XhoI* site of pBMN-IRES-GFP. pcDNA3-p38AGF was a gift of Roger Davies (University of Massachusetts, Worcester, MA). Plasmids phCMV-VSVG, encoding vesicular stomatitis virus glycoprotein (VSV-G), and pCMVgag-pol containing the Moloney murine leukemia virus (MoMLV) gag and pol genes were provided by Jane Burns (University of California at San Diego).

Retroviral infection of cells. Retroviruses were prepared by transfection of HEK-293T cells with 15 μ g DNA/100mm dish of three plasmids encoding gag/pol, VSV-G, and the target construct, ratio

4:3:8. Supernatants from cells were collected for three days and combined, filtered through 0.4- μ m filters, and stored in aliquots at -80°C . NMuMG cells were infected with supernatant containing retroviruses in the presence of 6 $\mu\text{g/ml}$ Polybrene (Sigma) as described previously (Yee et al., 1994). Three days later, GFP-positive cells were selected by flow cytometry. Under these conditions more than 95% of selected cells expressed GFP at the time of experiments.

Immunoblot Analysis. Cells were incubated in serum-free medium for 4 h prior to treatment with TGF β 1. Cells were lysed in buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 $\mu\text{g/ml}$ aprotinin, and 2 $\mu\text{g/ml}$ leupeptin. Protein concentrations in cell lysates were determined by the Bradford method. Protein extracts (50 $\mu\text{g/lane}$) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (100 mA, 2.5 h). Membranes were blocked with 5% milk in TBST for 1 h at room temperature (RT) and then incubated with primary antibodies in TBST plus 1% milk for 16 h at 4°C , followed by incubation with secondary antibodies for 1 h at RT. Membranes were washed 3 times in TBST and immunoreactive bands visualized by ECL (Pierce).

p38Mapk *in vitro* kinase assay. p38Mapk was precipitated from protein extracts (200 μg) with a p38Mapk monoclonal antibody (Santa Cruz Biotechnology, Inc.) for 2 h at 4°C . An *in vitro* kinase reaction was performed in a 40- μl volume by adding to the immune complexes 1 μg GST-ATF2 and 10 μCi γ -[^{32}P]-ATP (specific activity 3000 Ci/mmol, New England Nuclear) for 20 min at 30°C in the presence of 10 μM PKA peptide inhibitor (Calbiochem). Reactions were terminated by addition of Laemmli loading buffer and heating, followed by 15% SDS-PAGE and transfer to nitrocellulose (NC) membranes. Quantitative analysis of [^{32}P]-labeled bands was performed using a

PhosphorImager (Molecular Dynamics). The same NC-membranes were probed with a monoclonal antibody to p38Mapk.

Immunofluorescence microscopy. NMuMG cells (10^5 cells/well) were grown in DMEM containing 5% FBS on glass cover-slips (22x22 mm) for 24 h before treatment with 2 ng/ml TGF β 1. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at RT and then permeabilized with 0.05% Triton X-100 for 10 min. Cells were washed three times in PBS after each treatment. Cells were blocked with 3% milk in PBS for 30 min at RT, incubated for 60 min with primary antibodies diluted in 1% milk/PBS (1/300 for ZO-1, 1/500 for Smad2, 1/250 for fibronectin), and then with fluorescent secondary antibodies (1/500) for 45 min at RT. Microtubules were stained for 30 min at RT with β -tubulin-Cy3 diluted 1/250 in 1% milk/PBS. Actin was stained with phalloidin-FITC (4 units/ml) or phalloidin-Texas Red (2 units/ml). Cell nuclei were stained with 1 μ g/ml Hoechst for 10 min at RT. Cover-slips were mounted on 25x75-mm microslides (VWR Scientific) using AquaPolyMount (Polysciences). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

Transcriptional Assays. NMuMG cells (3×10^4) were seeded in 24-well plates and transfected with 0.16 μ g/ml pSBE-Lux containing twelve repeats of Smad binding sequence (provided by J.-M. Gauthier, Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) with 0.002 μ g/ml pCMV-Rl (Promega, Madison, WI) using FuGENE6 reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Cells were incubated for 8 h in 0.5% FBS-DMEM prior to treatment with 1 ng/ml TGF β 1 for 16 h. Firefly luciferase (Luc) and *Renilla reniformis* luciferase (RLuc) activities in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega)

according to the manufacturer's protocol in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luc activity was normalized to RLuc activity and presented as Relative Luciferase Units. All assays were done in triplicate wells and each experiment was repeated at least twice.

Affinity Precipitation of Rac using GST-PBD. A fusion protein containing the GTPase-binding domain from human PAK1 (PBD) and glutathione S-transferase (GST) was expressed in *Escherichia coli* using pGEX-4T3-GST-PBD as described in (Benard *et al.*, 1999). pGEX-4T3-GST-PBD was kindly provided by Dr. Gary Bokoch (Scripps Research Institute). NMuMG cells (2×10^7 /assay) were treated with 2 ng/ml TGF β 1 for 15 min followed by cell lysis in 20 mM Tris, pH 7.5, 150 mM NaCl, 5mM MgCl₂, 1% NP-40, 5% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 μ g/ml aprotinin, and 2 μ g/ml leupeptin in the presence of 8 μ g GT-PBD. Cell lysates were clarified by low speed centrifugation at 4°C. HEK293T cells transfected with Rac1N17 or Alk5 mutants were lysed in the same buffer. After clarification, cell lysates (350 μ g/assay) were incubated with 8 μ g GST-PBD. To prepare cytosolic Rac1 loaded with GDP or GTP γ S, cell lysates (equivalent of 2×10^6 cells) were incubated for 15 min at 30°C in the presence of 10 mM EDTA and 100 mM GTP γ S or 1 mM GDP to facilitate nucleotide exchange (Benard *et al.*, 1999). The loading reaction was terminated by addition of 60 mM MgCl₂. Affinity precipitation was performed using 15 μ l of glutathione-Sepharose 4B beads (Pharmacia) for 1 h at 4°C. The bead pellets were washed three times with 20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% NP-40 and 2 times in PBS. The bead pellet was finally suspended in 40 μ l of Laemmli sample buffer. Proteins were separated on 15% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with an antibody to Rac1 (Transduction Laboratories).

Migration Assays. NMuMG or MDA-MB-231 cells (1×10^5 /well) were plated in DMEM/0.5%FBS in the upper chamber of 5- μ m pore (24-well) transwells (Costar, High Wycombe Bucks, UK) and incubated alone or with 2 ng/ml TGF β 1 in the absence or presence of SB202190. After 16 h, cells were fixed in 100% methanol and cells remaining at the top of the polycarbonate membrane were removed with cotton swabs. The cells that had migrated through pores to the lower surface were stained with Diff-quick stain (VWR Scientific). Membranes were mounted on 25x75-mm microslides. Four random images were recorded at 200X magnification and cells were counted. Experiments were performed in duplicates.

Wound closure assay. MDA-MB-231 and 4T1 cells ($1-2 \times 10^5$ /well) were seeded in 12-well plates. Cells were incubated in serum-free medium for 32 h prior to wounding. The wounds were made by scraping with plastic tip across the cell monolayer. Cells were treated with kinase inhibitors 60 min before wounding. The wounded cells were treated or untreated with 2 ng/ml TGF β 1. Phase contrast images were recorded at the time of wounding (0 h) and 16 h thereafter. The wound closure was estimated as the ratio of the remaining wound area relative to the initial wounded area. Experiments were repeated at least three times.

RESULTS

H-7 inhibits TGF β -mediated activation of p38Mapk and EMT. We investigated TGF β -mediated EMT in NMuMG mouse mammary epithelial cells. These mammary epithelial cells have cuboidal cell shape and form tight and adherens junctions. Treatment with 2 ng/ml TGF β for 24 h induced changes in the cell morphology from cuboidal to an elongated spindle-like shape (Figure 1A). Consistent with previous studies (Miettinen et al., 1994), TGF β -mediated EMT was blocked in the

presence of 20 μ M H-7 (Figure 1A). The inhibitors were added 60 min prior to addition of TGF β and were present during a complete duration of the experiment. Although H-7 has been introduced as a protein kinase C inhibitor, it can inhibit other kinases including PKA and PKG (Quick et al., 1992). Therefore, we investigated the effect of H-7 on the signaling pathways induced by TGF β . We found that TGF β 1-induced phosphorylation of Smad2 was not affected by the presence of H-7 at the concentration that block EMT (Figure 1C). Inhibition of JNK and ERK1/2 did not affect EMT (Bakin et al., 2000; Bhowmick et al., 2001a). Therefore, we tested whether H-7 affects TGF β -mediated activation of the p38Mapk pathway using polyclonal antibodies to phosphorylated (active) MKK3/6 and p38Mapk. TGF β -mediated phosphorylation of p38Mapk was blocked in the presence of 20 μ M H-7 (Figure 1C). H-7 also inhibited TGF β -induced phosphorylation of ATF2, a substrate of p38Mapk (Figure 1D).

Next, we checked whether H-7 inhibits activation of MKK3/6. We found that TGF β -induced phosphorylation of MKK3/6 was inhibited by H-7 in a dose dependent manner (Figure 1E), suggesting that H-7 inhibits a kinase upstream of MKK3/6. This kinase is downstream of TGF β receptors as incubation with 5 to 40 μ M H-7 did not block phosphorylation of Smad2 (Figure 1B). Consistent with this result, H-7 did not block TGF β -mediated activity of Smad-dependent luciferase reporter (Figure 1F). Since H-7 can inhibit PKC, we examined activation of p38Mapk in the presence of another PKC inhibitor, bisindolylmaleimide-I (BIM-I) (Davies et al., 2000). Treatment of cells with doses of BIM-I (1-5 μ M) that block typical PKCs (Davies et al., 2000) did not affect phosphorylation of MKK3/6 in response to TGF β (Figure 1D). These results suggest that H-7 impairs TGF β signaling by inhibiting activation of the p38Mapk pathway downstream of TGF β receptors, and not through its effect on PKCs.

p38Mapk is involved in TGF β -mediated EMT. To test whether p38Mapk is involved in EMT, we used specific inhibitors of p38Mapk, SB202190 and SB203580, that do not affect JNK, MEK1/2 and ERK1/2 (Davies et al., 2000). Microscopic examination showed that cell elongation induced by TGF β in NMuMG cells was blocked by co-treatment with 10 μ M SB202190 (Figure 2A). Similarly, the p38Mapk inhibitor blocked TGF β -induced cell elongation in cervical cancer epithelial SiHa cells (Figure 2A). Previous studies have shown that these p38Mapk inhibitors may affect the kinase activity of TGF β receptors (Eyers et al., 1998). Therefore, we examined their effect on TGF β -receptor dependent phosphorylation of Smad2. Treatment of cells with TGF β in the presence of SB202190 did not significantly affect the expression and TGF β -induced phosphorylation of Smad2 (Figure 2B), whereas it reduced phosphorylation of ATF2 (Figure 2C). Similar results were obtained with SB203580 (data not shown).

TGF β activates the p38Mapk pathway in NMuMG and SiHa cells. We next examined activation of the p38Mapk pathway in response to TGF β . Protein extracts were prepared from cells starved in serum-free medium for 4 h and treated with TGF β 1. Phosphorylation of MKK3/6 was detected after 15 min of TGF β treatment reaching a maximum at 60 min, whereas an increase in p38Mapk phosphorylation at Thr180/Tyr182 was observed at 30 min and reached a plateau at 60 min (Figure 3A). To confirm the immunoblot data, we tested p38Mapk specific activity using an *in vitro* kinase assay with GST-ATF2 fusion protein as substrate. Treatment with TGF β increased 32 P incorporation onto GST-ATF2 in a time-dependent fashion, 6-fold at 15 min and reaching a maximal stimulation of 24-fold above control by 60 min (Figure 3B). This increase in p38Mapk kinase activity at 15 min may reflect a higher sensitivity of the *in vitro* kinase assay compare to detection of phosphorylated p38Mapk by immunoblot. TGF β -induced activation of p38Mapk was dose-dependent with 0.1 ng/ml

being sufficient to induce phosphorylation of p38Mapk with a maximal effect observed between 0.5 and 2 ng/ml (Figure 3C). Treatment of SiHa human cervical carcinoma cells with TGF β 1 for 60 min resulted in phosphorylation of p38Mapk (Figure 3D), suggesting activation of p38Mapk signaling in response to TGF β 1 in these cells.

Kinase activities of TGF β receptors are required for TGF β -induced p38Mapk activation. To confirm the role of TGF β receptors in activation of p38Mapk, we engineered cells expressing T β RII-K277R, a kinase-inactive mutant of TGF β type II receptor (Wrana et al., 1994). NMuMG cells were infected with retrovirus encoding T β RII-K277R and enhanced green fluorescent protein (EGFP) or with control retrovirus encoding EGFP only (Gabe). Fluorescent cells were selected by flow cytometry and expression of the HA-tagged mutant receptor was confirmed by immunoblot analysis (Figure 4A). TGF β -mediated phosphorylation of Smad2, MKK3/6, and p38Mapk was inhibited in T β RII-K277R cells compared to control Gabe cells (Figure 4B). T β RII-K277R also blocked EMT (Figure 4D) and cell migration (Figure 8A), indicating that T β RII kinase activity is required for these TGF β responses.

To determine whether the activation of p38Mapk was TGF β -specific, we expressed wild-type T β RI/Alk5 (Alk5-WT), kinase-inactive Alk5-K232R, or kinase active Alk5-T204D (Kawabata et al., 1995) in NMuMG cells. Alk5 mutants were expressed using a bi-cistronic retroviral vector encoding EGFP. GFP-positive cells were selected by flow cytometry and expression of mutants was confirmed by immunoblot analysis (Figure 4B). Kinase-inactive Alk5-K232R significantly reduced TGF β -induced phosphorylation of MKK3/6 and p38Mapk, whereas kinase active Alk5-T204D induced phosphorylation of MKK3/6 and p38Mapk in the absence of added ligand (Figure 4B). Microscopic studies showed that TGF β -induced EMT was impaired in cells expressing kinase-

inactive Alk5-K232R. Cells expressing Alk5-T204D exhibited a fibroblastic morphology similar to Alk5-WT cells treated with TGF β for 24 h (Figure 4C). Treatment of cells expressing Alk5-T204D with the p38Mapk inhibitor SB202190 reversed these morphological changes.

MKK3/6 kinases mediate activation of p38Mapk and EMT in response to TGF β . Dual-specificity MKK3 and MKK6 kinases have been implicated in activation of p38Mapk (Raingeaud et al., 1996). Phosphorylation of both kinases is induced by TGF β or by expression of active Alk5-T204D in NMuMG cells (Figure 4B). Therefore, we tested the effect of dominant-negative MKK3AL (Huang et al., 1997; Zanke et al., 1996) on TGF β -mediated activation of p38Mapk and EMT in NMuMG cells. Expression of HA-tagged MKK3AL reduced phosphorylation of endogenous p38Mapk and ATF2 (Figure 5A), whereas expression and phosphorylation of Smad2 were not affected (Figure 5B). Similar results were obtained with dominant negative MKK6AL (data not shown). Next, we examined effect of MKK3AL on EMT. TGF β induced EMT in NMuMG cells infected with control retrovirus encoding EGFP only (BMN), whereas EMT was inhibited in MKK3AL expressing cells (Figure 5C). SB202190, a p38Mapk inhibitor, blocks activity of p38 α and p38 β but does not inhibit p38 γ and p38 δ (Davies et al., 2000). Since, SB202190 blocked EMT (Figure 2A), we tested the effect of p38AGF, a dominant negative mutant of p38 α , on TGF β -mediated EMT. TGF β -induced morphological transformation in NMuMG infected with retroviruses encoding p38AGF was impaired compared to cells infected with control BMN virus (Figure 5C). These findings suggest that MKK3/6 kinases mediate TGF β -induced activation of p38Mapk and EMT in NMuMG cells.

p38Mapk is involved in TGF β -induced reorganization of the actin cytoskeleton. We characterized the effect of p38Mapk inhibitors on reorganization of the actin cytoskeleton in response to TGF β . Microscopic examination of F-actin by staining with phalloidin-fluorescein showed a cortical arrangement of actin at the cell-cell junctions without significant stress fibers (Figure 6A). Treatment with TGF β 1 for 24 h induced formation of actin stress fibers arranged along the largest cell axis. SB202190 did not significantly change the actin organization in TGF β -untreated cells, but impaired TGF β -induced formation of actin stress fibers (Figure 6A). Similar blockade of stress fiber formation was observed in cells pretreated with H-7 (data not shown). Examination of the actin cytoskeleton in MKK3AL cells showed that MKK3AL did not affect the cortical arrangement of actin in untreated cells, but inhibited TGF β -induced actin stress fiber formation (Figure 6B). These data suggest that p38Mapk contributes to the reorganization of the actin cytoskeleton induced by TGF β during EMT.

Rac GTP-binding protein is involved in TGF β -induced activation of p38Mapk and EMT.

There is evidence that small GTP-binding proteins are involved in TGF β signaling (Atfi et al., 1997; Bakin et al., 2000; Bhowmick et al., 2001a; Engel et al., 1999; Mucsi et al., 1996). Rac1 and CDC42 have been implicated in the activation of the MKK3/6-p38Mapk cascade in several systems (Coghlan et al., 2000; Tibbles et al., 1996; Uddin et al., 2000; Zhang et al., 1995). To test whether Rac1 or RhoA are involved in p38Mapk activation in response to TGF β , we transfected dominant negative RhoAN19 or Rac1N17 in NMuMG cells. Rac1N17 inhibited TGF β 1-induced phosphorylation of p38Mapk and its downstream substrate ATF2, whereas neither dominant negative RhoAN19 nor constitutively active RhoAQ63L did not affect p38Mapk phosphorylation (Figure 7A,B). These data suggest that Rac1 mediates p38Mapk activation in response to TGF β .

To examine whether Rac1 activity is induced by TGF β , we performed affinity precipitation assay using a fusion protein of the GTPase-binding domain (aminoacids 67-152) from human PAK1 (PBD) and GST. The GST-PBD fusion protein has been shown to specifically bind active Rac1 loaded with GTP (Benard *et al.*, 1999). Treatment of NMuMG cells for 15 min with TGF β resulted in the increase in Rac1 binding to purified GST-PBD (Figure 7C). GST-PBD effectively interacted with the active GTP γ S-bound form of Rac1 but did not bind to the inactive GDP-bound form of Rac1 (Figure 7C, left inset). To confirm that TGF β receptors can mediate activation of Rac1, we expressed mutants of Alk5-T β RI in HEK293T cells. Kinase-inactive Alk5K232R reduced the level of active Rac1, whereas kinase active Alk5T204D increased the amount of Rac1 bound to GST-PBD. Expression of dominant negative Rac1N17 reduced the amount of Rac1 recovered from GST-PBD beads (Figure 7D). Since active Rac1 mediates actin ruffling and lamellipodia formation (Hall, 1998), we examined F-actin in NMuMG and MDA-MB-231 cells treated with 2 ng/ml of TGF β 1 for 15 min. Confocal microscopy of cells stained with phalloidin-Texas Red showed that TGF β induced actin ruffles, a phenotype associated with active Rac (Figure 7E).

In order to examine the role of Rac1 in EMT, NMuMG cells were infected with a retrovirus encoding dominant negative Rac1N17 and Green Fluorescent Protein (GFP). Immunoblot analysis showed at least two-fold higher level of Rac1 in cells infected with Rac1N17 retrovirus compared to cells infected with control BMN virus encoding GFP only (Figure 8A). TGF β induced phosphorylation of MKK3/6 and p38Mapk in cells infected with control retrovirus, whereas, in Rac1N17 cells, this induction was significantly reduced (Figure 8B). Rac1N17 did not significantly affect TGF β -dependent phosphorylation of Smad2 (Figure 8C). Microscopic examination showed that TGF β 1 induced cell elongation and the formation of actin stress fibers in control BMN cells,

whereas these effects were impaired in cells expressing Rac1N17 (Figure 8D). These findings suggest that Rac1 is involved in TGF β -induced EMT and activation of p38Mapk.

p38Mapk inhibitors block TGF β -mediated cell motility. TGF β stimulates chemotaxis and migration of tumor and nontumor cells (Ashcroft et al., 1999; Postlethwaite et al., 1987). Recent studies implicated p38Mapk in TGF β -induced chemotaxis of human neutrophils (Hannigan et al., 1998). We next tested effect the p38Mapk inhibitors on TGF β mediated migration of NMuMG (nontumor) and MDA-MB-231 (tumor) cells. TGF β stimulated approximately 3-fold the chemotactic migration of NMuMG cells through polycarbonate filters (Figure 9A). Migration of NMuMG cells was significantly inhibited by SB202190 as well as in NMuMG cells infected with kinase-inactive TGF β type II receptor (T β R II -K277R) compared to the those infected with control Gabe retrovirus (Figure 9A). TGF β stimulated approximately 6-fold migration of breast cancer MDA-MB-231 cells. This was also blocked by SB202190 (Figure 9B).

To investigate further the role of p38Mapk in TGF β -mediated cell migration, wounds were made in confluent cultures of MDA-MB-231 and 4T1 breast cancer cells. These cells are not growth inhibited by TGF β 1. Addition of TGF β 1 to serum-free medium accelerated the wound closure in both cell lines, whereas in the presence of the p38Mapk inhibitor the wounds stayed opened (Figure 9D). These data suggest that p38Mapk is involved in TGF β -induced cell migration.

DISCUSSION

TGF β can induce mesenchymal transdifferentiation in epithelial and endothelial cells (Boyer et al., 1999; Brown et al., 1999; Miettinen et al., 1994). Early studies have shown that protein kinase inhibitor H-7 blocks TGF β -induced EMT but a particular signaling cascade affected by H-7 was not

identified (Miettinen et al., 1994). We found that H-7 inhibited TGF β -induced phosphorylation of MKK3/6 kinases, but did not affect phosphorylation of Smad2 and Smad-dependent transcriptional responses. These results suggest that H-7 affects a kinase that mediates signaling downstream of TGF β receptors, but upstream of MKK3/6 kinases. This kinase is distinct from typical PKCs, since BIM-I, an inhibitor of typical PKCs, did not block TGF β -induced EMT and phosphorylation of MKK3/6 and p38Mapk. The candidate kinases include atypical PKCs and kinase(s) implicated in activation of MKK3/6 such as PAK1 (Zhang et al., 1995), TAK1 (Yamaguchi et al., 1995), and MLK3 (Tibbles et al., 1996).

The H-7 studies suggested a critical role for the p38Mapk pathway in EMT. This hypothesis was further tested using the p38Mapk specific inhibitors, SB202190 and SB203580, which do not inhibit JNK, MEK1/2 and ERK1/2 kinases (Davies et al., 2000). SB202190 and SB203580 blocked TGF β -induced cell morphological changes in NMuMG mouse mammary epithelial cells and SiHa human cervical carcinoma cells. The p38Mapk inhibitors blocked TGF β -induced phosphorylation of ATF2, a p38Mapk substrate, without effect on Smad2 phosphorylation, implying that under these experimental conditions the blockade of p38Mapk did not affect TGF β receptor kinase activity.

To test whether activation of p38Mapk by TGF β is a direct event, we investigated the kinetics of activating phosphorylation of MKK3/6 and p38Mapk. TGF β induced phosphorylation of Smad2 and MKK3/6 kinases with similar kinetics (15 min). Phosphorylation of p38Mapk was delayed (30 min) suggesting that this event requires activation of MKK3/6. We further showed that dominant negative mutants of MKK3 and MKK6 interfering with p38Mapk activation (Raingaud et al., 1996) impaired TGF β -induced phosphorylation of p38Mapk and ATF2, indicating that the MKK3/6-p38Mapk module mediates TGF β signaling in NMuMG cells. The dose-dependent

increase in p38Mapk activity was confirmed by *in vitro* kinase assay and by phosphorylation of ATF2.

To confirm the specificity of TGF β signaling to p38Mapk we performed studies with TGF β receptor mutants. Kinase-inactive type II receptor blocked EMT and phosphorylation of Smad2 as well as MKK3/6 and p38Mapk, indicating that kinase function of T β RII is required for activation of p38Mapk and EMT. Kinase-inactive T β RI/Alk5-K232R also blocked TGF β -induced activation of the p38Mapk pathway, whereas expression of kinase active Alk5-T204D resulted in phosphorylation of MKK3/6 and p38Mapk and EMT in the absence of added TGF β 1. Thus, kinase activities of both TGF β receptors are required for TGF β -induced activation of the p38Mapk pathway, and Alk5-T204D can signal to p38Mapk in the absence of added ligand. Alk5-T204D-induced EMT was inhibited by SB202190, a p38Mapk inhibitor, suggesting that p38Mapk mediates EMT induced by Alk5-T204D.

Activation of p38Mapk is mediated by Rac1/CDC42 GTP-binding proteins (Coghlan et al., 2000; Tibbles et al., 1996; Uddin et al., 2000; Zhang et al., 1995). Small GTP-binding proteins are also involved in TGF β responses (Atfi et al., 1997; Bakin et al., 2000; Bhowmick et al., 2001a; Engel et al., 1999; Mucsi et al., 1996). We found that dominant negative Rac1N17 impaired activation of the p38Mapk pathway in NMuMG cells, whereas RhoAN19 did not block this event. Expression of Rac1N17 did not affect phosphorylation of Smad2. These data suggest that Rac1 mediates TGF β -induced p38Mapk activation independently of Smad activation. The mechanism of downstream signaling events is unclear. Previous studies showed that PAK1 mediates p38Mapk activation downstream of Rac1 and CDC42 (Zhang et al., 1995). On the other hand, TGF β -activated kinase 1, TAK1, has been implicated in p38Mapk activation in response to BMP and TGF β in several cell systems (Yamaguchi et al., 1995).

Expression of dominant negative Rac1N17 in NMuMG cells inhibited TGF β 1-induced changes in cell shape and the actin cytoskeleton suggesting involvement of Rac1 in TGF β -induced EMT. This result is consistent with other reports. For example, both D-Rac and D-p38 have been reported to contribute to Dpp signaling during wing morphogenesis in *Drosophila* (Adachi-Yamada et al., 1999; Eaton et al., 1995). There is also evidence that Rac1 is required for EMT induced by hepatocyte growth factor (HGF) in MDCK cells (Ridley et al., 1995; Royal et al., 2000). Dominant negative Rac/CDC42 mutants inhibit oncogenic Ras-induced cell transformation (Qiu et al., 1997; Qiu et al., 1995), and Ras has been shown to cooperate with TGF β in the induction of EMT (Oft et al., 1996). In addition, Rho/Rac/CDC42 proteins are involved in morphogenesis by regulating the actin cytoskeleton (Hall, 1998). Therefore, Rac1 may contribute to TGF β -induced EMT via its effects on the cell cytoskeleton and/or via activation of the p38Mapk pathway. In NMuMG cells, TGF β 1 induced actin ruffles and activation of Rac1 within 15 min (Figure 7C,E). Expression of kinase-inactive Alk5K232R reduced, whereas constitutively active Alk5-T204D increased Rac1 loading with GTP (Figure 7D) and induced the formation of strong actin ruffles (data not shown). These results suggest that Rac activation and actin ruffling induced by TGF β may precede the formation of actin stress fibers, which does not occur until four hours after addition of TGF β 1 as it has been shown by Bhowmick et al. (Bhowmick et al., 2001a).

Inhibitors of p38Mapk and dominant negative MKK3AL impaired TGF β -induced changes in cell morphology and reorganization of the actin cytoskeleton. Expression of dominant negative mutant of p38 α also blocked TGF β -mediated EMT. Together, these results suggest that the p38Mapk pathway contributes to TGF β -induced alterations in the actin cytoskeleton and the cell shape during EMT. Consistent with this hypothesis, p38Mapk has been shown to mediate regulation of the actin cytoskeleton in smooth muscle myocytes in response to TGF β (Hedges et al., 1999), and

in H₂O₂-induced rapid reorganization of the actin cytoskeleton in endothelial and mesenchymal cells (Huot et al., 1998). A recent study reported involvement of p38Mapk in TGF β -mediated EMT (Bhowmick et al., 2001b). In this report, adenoviral transduction of dominant negative p38 β inhibited TGF β -mediated EMT at the step of disruption of junctional complexes but did not alter actin reorganization. We found that p38Mapk inhibitors and dominant negative MKK3AL affected actin stress fiber formation (Figure 6). TGF β and Alk5T204D activated both MKK3 and MKK6 in NMuMG cells (Figure 3,4). This suggests that TGF β may activate multiple p38Mapk isoforms in NMuMG cells as MKK3 preferentially activates p38 α and p38 γ while MKK6 activates p38Mapks α , β , and γ (Enslen et al., 1998). Recent studies showed that p38 α and p38 β may have different functions (Wang et al., 1998) and different subcellular localization (Lee et al., 2000). p38Mapk inhibitors block activity of both p38 α and p38 β (Enslen et al., 1998) and MKK3AL impaired phosphorylation of p38Mapk in NMuMG cells as measured with an antibody that recognizes both α and β isoforms. Therefore, multiple p38Mapks may be involved in TGF β -induced EMT and mediate different aspects of EMT, potentially explaining the discrepancies with the paper by Bhowmick *et al.*

EMT is a complex process involving restructuring the cell cytoskeleton, cell membrane, and cell-cell junctions. Previous studies implicated several molecules in different aspects of EMT. Smad transcription factors have been shown to synergize with Alk5 in induction of EMT but no specific function has been associated with these factors (Piek et al., 1999a). PI3K/Akt may contribute to dissolution of tight junctions and to TGF β transcriptional responses (Bakin et al., 2000). RhoA/Rock signaling has been implicated in the actin stress fiber formation (Bhowmick et al., 2001a). What aspect of EMT can be mediated by p38Mapk? p38Mapk can regulate the actin organization via HSP27 (Hedges et al., 1999; Huot et al., 1998). Therefore, p38Mapk may function in TGF β -induced

reorganization of the actin cytoskeleton in parallel or upstream of the RhoA/Rock pathway since dn-RhoA and Y27632, a Rock kinase inhibitor, did not affect activation of p38Mapk by TGF β (data not shown). In addition, p38Mapk may contribute to the expression of TGF β target genes that are casually involved in EMT as p38Mapk has been implicated in TGF β -transcriptional responses by activating ATF2 and Sp1 (Park et al., 2000; Raingeaud et al., 1996; Sano et al., 1999).

Finally, we investigated the role of p38Mapk in TGF β -induced migration of mouse and human mammary epithelial cells. The p38Mapk inhibitors blocked TGF β -stimulated migration of NMuMG, MDA-MB-231, and 4T1 cells. These results are consistent with the proposed role of p38Mapk in TGF β -mediated chemotaxis of human neutrophils (Hannigan et al., 1998) and smooth muscle cells (Hedges et al., 1999). Interestingly, Smad3-deficient keratinocytes and monocytes are impaired in chemotactic response to TGF β (Ashcroft et al., 1999) whereas p38Mapk inhibitors did not affect Smad2 phosphorylation (Figure 2). These data suggest that the p38Mapk pathway may act in parallel or in cooperation with a Smad-dependent pathway in chemotactic responses to TGF β . The data presented suggest that p38Mapk signaling plays a critical role in TGF β -induced EMT and cell migration. This pathway may be considered as a potential target of therapeutic interventions in neoplastic and inflammatory disorders associated with TGF β -mediated EMT.

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ABBREVIATIONS

BMP, bone morphogenic protein; ERK, extracellular signal-regulated kinase; HA, haemagglutinin; JNK, Jun N-terminal kinase; Mapk, mitogen-activated protein kinase; MKK, MAP kinase kinase; PI3K, phosphatidylinositide-3 kinase; PKC, protein kinase C; TGF β , transforming growth factor β ; T β RII, TGF β type II receptor; T β RI, TGF β type I receptor; TNF α , tumor necrosis factor α ;

FIGURE LEGENDS

Figure 1. Inhibition of TGF β -mediated EMT and p38Mapk activation by H-7 kinase inhibitor.

(A) NMuMG mammary epithelial cells were grown on glass coverslips for 24 h and treated (bottom row) or not (top row) with 2 ng/ml TGF β 1 for 24 h. Where indicated, cells were co-incubated with 20 μ M H-7. Phase contrast images were taken at 200x magnification. (B-E) Immunoblot analysis of whole-cell extracts from NMuMG cells treated with 2 ng/ml TGF β 1 for the indicated times. Kinase inhibitors were added 60 min prior TGF β treatment. (B) Immunoblot detection of phospho-Smad2 and total Smad2. (C) Detection of phospho-p38Mapk total p38Mapk. (D) Inhibition of TGF β -induced ATF2 phosphorylation by H-7. Immunoblots with antisera to phospho-ATF2 and total ATF2. (E) TGF β -induced phosphorylation of MKK3/6 in cells co-treated with various concentrations of H-7 or 5 μ M BIM-I, a PKC inhibitor. (F) Luciferase activity in NMuMG transfected with Smad-dependent reporter pSBE-Lux and pCMV-Rl vectors and treated with 1 ng/ml TGF β 1 for 16 h in the absence or presence of 20 μ M H-7. Each bar represents the mean \pm SD of three wells.

Figure 2. Blockade of TGF β -induced EMT by SB202190. (A) NMuMG cells grown on glass coverslips were treated (bottom row) or not (top row) with 2 ng/ml TGF β 1 for 24 h in the absence or presence of 10 μ M SB202190. Phase contrast images were taken at 200x magnification. (B) phospho-Smad2 and total Smad2 immunoblot analysis of whole-cell extracts from cells treated with 2 ng/ml TGF β 1 in the absence or presence of SB202190. (C) Immunoblots with antisera to phospho-ATF2 and total ATF2. SB202190 inhibits TGF β -induced phosphorylation of ATF2.

Figure 3. Activation of the p38Mapk pathway in response to TGF β . NMuMG cells were

incubated in serum-free medium for 4 h prior addition of TGF β 1. (A) Immunoblot analyses with antibodies to phospho-Smad2, phospho-MKK3/6, phospho-p38Mapk and total Smad2, MKK3/6, and p38Mapk. (B) Detection of p38Mapk kinase activity in whole-cell extracts from NMuMG cells treated with 2 ng/ml TGF β 1 using GST-ATF2 as substrate. The products were separated by SDS-PAGE and transferred onto nitrocellulose-membrane. 32 P incorporation onto ATF2 was quantitated using PhosphorImager. The membrane was probed with antibody to p38Mapk. (C) Immunoblot detection of TGF β 1 dose-dependent effect on p38Mapk phosphorylation at 60 min in NMuMG cells. (D) Induction of p38Mapk phosphorylation by 2 ng/ml TGF β 1 at 60 min in SiHa cells.

Figure 4. Effect of kinase mutant TGF β receptors on TGF β -induced EMT and activation of the p38Mapk pathway. (A) Immunoblot analyses of whole-cell extracts from NMuMG cells infected with retrovirus encoding T β RII-K277R or control virus (Gabe). Cells were treated with 2 ng/ml TGF β 1 for 60 min. Expression of HA-tagged T β RII-K277R was detected with antisera to the HA-epitope. Dominant negative T β RII-K277R inhibits phosphorylation of Smad2, MKK3/6, and p38Mapk in response to TGF β . Membranes were re-probed with antibodies to total Smad2 and p38Mapk. (B) Immunoblot analyses of whole-cell extracts from NMuMG cells infected with retroviruses encoding HA-tagged wild-type (WT) T β RI/Alk5, kinase-inactive Alk5-K232R, and kinase-active Alk5-T204D. Cells were treated with 2 ng/ml TGF β 1 for 60 min, and protein extracts were probed with antibodies to phospho-MKK3/6, phospho-p38Mapk, and total p38Mapk. Membranes were re-probed with antisera to the HA-epitope. (C) Phase contrast images of NMuMG cells expressing wild type Alk5-WT, Alk5-K232R, and T β RII-K277R. Cells grown on glass coverslips were untreated (top row) or treated (bottom row) with 2 ng/ml TGF β 1 for 24 h. (D)

NMuMG cells expressing Alk5-T204D were untreated or treated with 15 μ M SB202190 for 24 h. Phase contrast images were recorded at 200x magnification.

Figure 5. Effect of dominant negative MKK3AL and p38AGF on TGF β -mediated EMT. (A) Immunoblot analysis of p38Mapk and ATF2 phosphorylation in NMuMG cells transfected with empty vector (BMN) or plasmid encoding HA-tagged MKK3AL. Thirty-six hours after transfection cells were treated with 2 ng/ml TGF β 1 for 60 min. Whole-cell extracts were probed with phospho-specific antisera, and re-probed with antisera to total protein. Expression of MKK3AL was detected with antisera to the HA-epitope. **(B)** Immunoblot detection of Smad2 phosphorylation in MKK3AL expressing cells. **(C)** Phase contrast images of NMuMG cells infected with control (BMN) retrovirus or retroviruses encoding dominant negative MKK3 (MKK3AL) or p38 α (p38AGF). Cells were untreated (top row) or treated with 2 ng/ml TGF β 1 for 24 h. Images were recorded at 200x magnification.

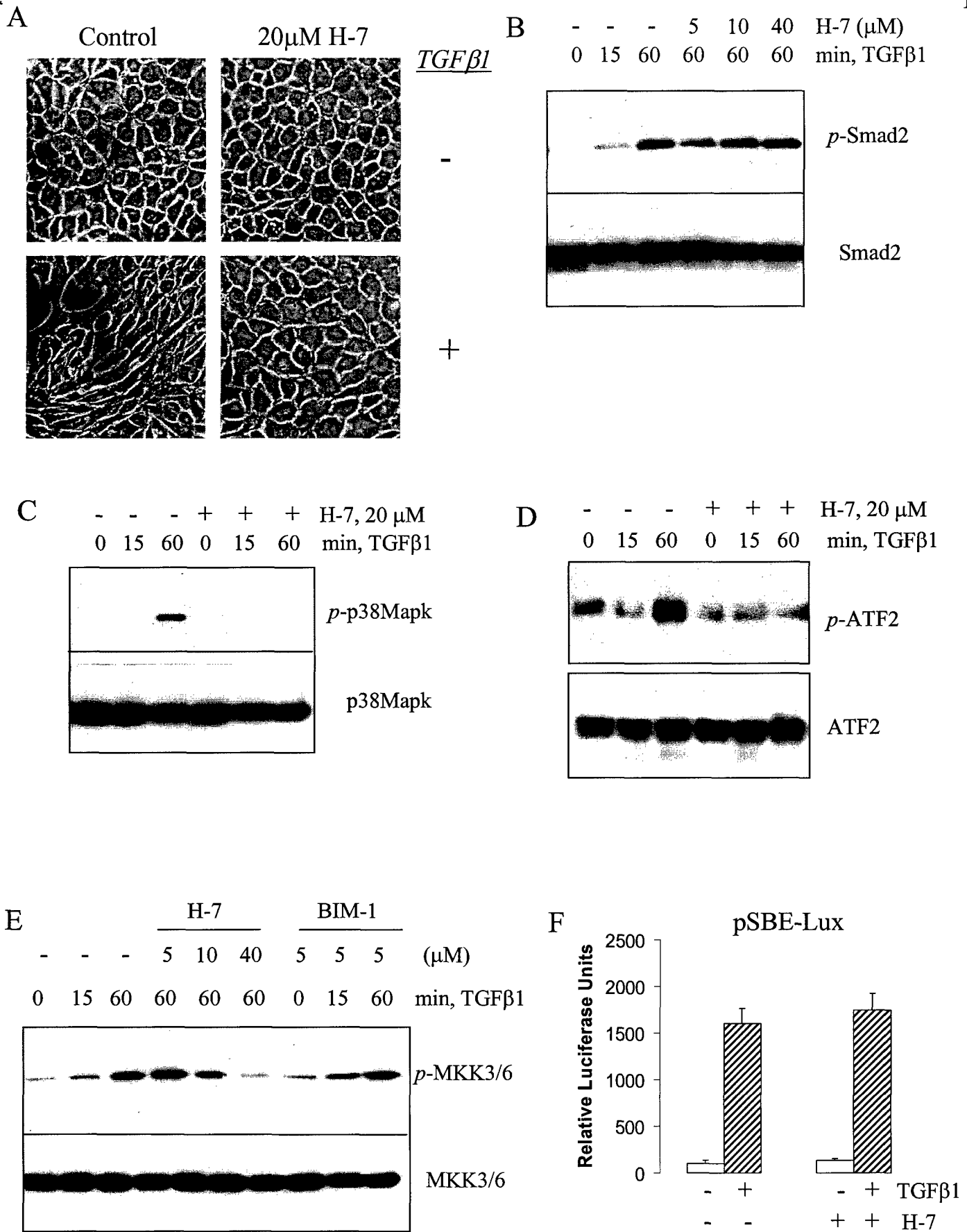
Figure 6. Effect of p38Mapk inhibitors on TGF β -induced reorganization of the actin cytoskeleton. (A) NMuMG cells grown on glass coverslips were incubated with 2 ng/ml TGF β 1 for 24 h in the absence or presence of 15 μ M SB202190. Cells were fixed and stained with phalloidin-Texas Red (actin). Actin staining and interference-contrast images (DIC) were recorded from the same cells. Note cell elongation and actin stress fibers formation in TGF β -treated cells in the absence of the p38Mapk inhibitor compared to cells treated with the p38Mapk inhibitor. **(D)** Actin cytoskeleton in NMuMG cells infected with MKK3AL or control (BMN) retroviruses. Cells were treated with 2 ng/ml TGF β 1 for 24 h and stained with phalloidin-Texas Red. Scale bars, 15 μ m.

Figure 7. Rac1 is involved in TGF β -mediated activation of p38Mapk. (A) Immunoblot analysis of p38Mapk and ATF2 phosphorylation in cells expressing Rac1N17 and treated with 2 ng/ml TGF β 1. (B) p38Mapk phosphorylation in cells expressing RhoAN19 or RhoAQ63L. (C) NMuMG cells were treated with 2 ng/ml TGF β 1 for 15 min. Cell lysates were clarified and used for affinity precipitation with 8 μ g of GST-PBD. Proteins bound to GST-PBD were separated on SDS-PAGE, transferred to nitrocellulose membrane and blotted with antibody to Rac1. The inset at the left shows the total signal detected using cell lysate pre-exchanged with either GTP γ S or GDP as described in "Experimental Procedures". (D) 293T cells were transfected with control plasmid (BMN), kinase-inactive Alk5K232R, kinase-active Alk5T204D or dominant negative Rac1N17. Cells were lysed 48 h after transfection. Cell lysates were clarified and used for affinity precipitation with 8 μ g of GST-PBD as described above. The bottom inset shows the Rac1 signal detected in total cell lysates. (E) Confocal images of F-actin in NMuMG cells treated with 2 ng/ml TGF β 1 for 15 min and stained with phalloidin-Texas Red. Arrows indicate the spots of actin polymerization at the cell edges.

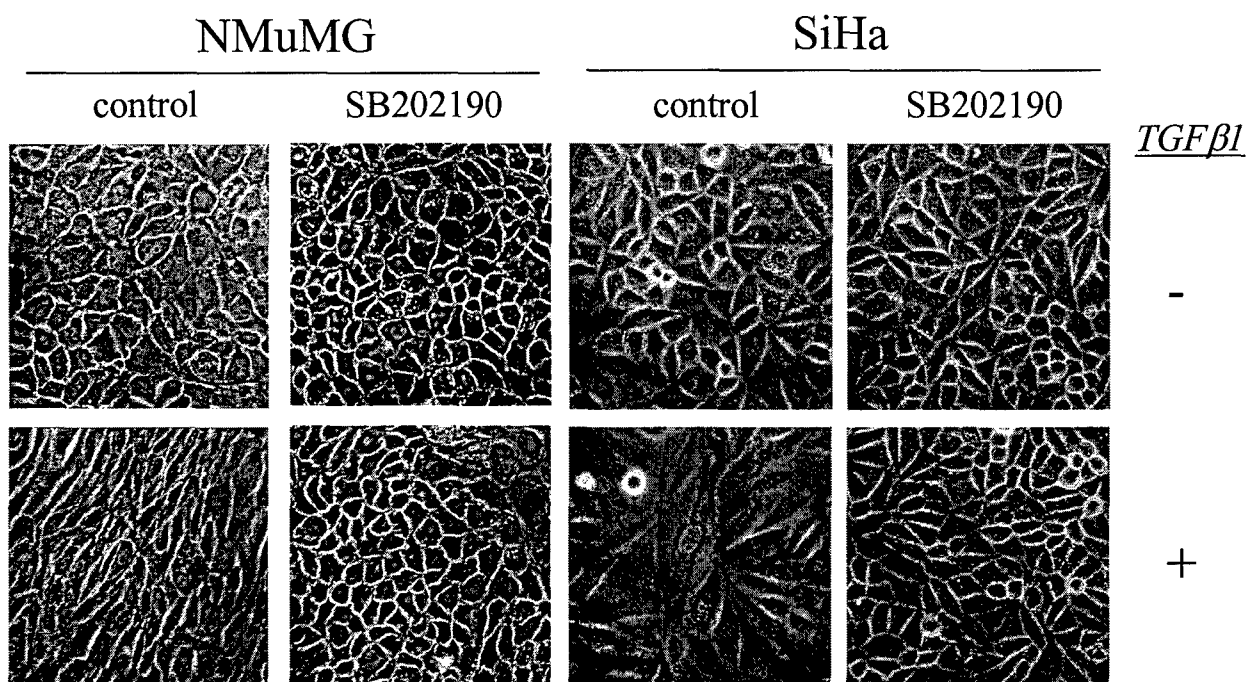
Figure 8. Dominant negative Rac1N17 blocks TGF β -mediated activation of p38Mapk and EMT. NMuMG cells were infected with retrovirus encoding Rac1N17 or control virus (BMN) and treated with 2 ng/ml TGF β 1. (A) Detection of Rac1N17 expression with antisera to Rac1. Cells infected with Rac1N17 show a higher level of Rac1 expression. (B) Immunoblot detection of MKK3/6 and p38Mapk phosphorylation in control (BMN) and Rac1N17 expressing cells. (C) Immunoblot with antisera to phospho-Smad2 and total Smad2. (D) Microscopic images from NMuMG cells infected with control retrovirus (BMN) or retrovirus encoding Rac1N17. Cells grown on glass cover-slips were treated with 2 ng/ml TGF β 1 for 24h. Cells were stained with phalloidin-Texas Red (actin). Actin and interference-contrast images (DIC) were recorded from the same cells.

Scale bar, 15 μ m.

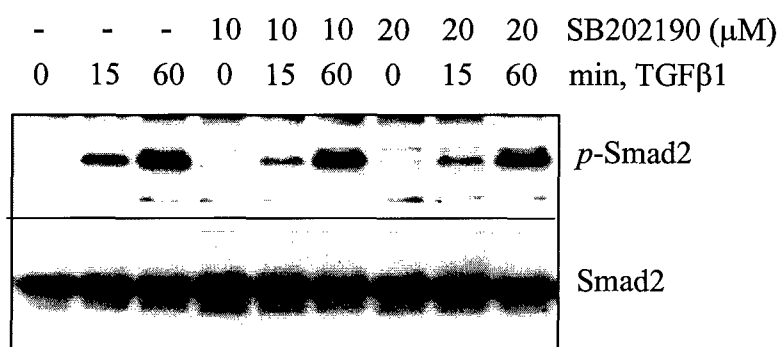
Figure 9. Involvement of p38Mapk in TGF β -mediated cell migration. (A, B) NMuMG or MDA-MB-231 cells (1×10^5 /well) were seeded in the upper chamber of 5- μ m pore transwells and 2 ng/ml TGF β 1 was added to the lower chamber. Cells were incubated for 16 h in the absence or presence of SB202190, a p38Mapk inhibitor. Cells migrating through pores were stained and counted from four random fields. Experiments were performed in duplicates. Values are the mean \pm SD of cells per field. Migration of NMuMG cells expressing kinase-inactive T β RII-K277R was compared to cells infected with control Gabe virus. (B) Blockade of MDA-MB-231 cell migration with 10 μ M SB202190. (C) Wound closure in monolayers of MDA-MB-231 and 4T1 cells following 16 h of treatment with 2 ng/ml TGF β 1 in the absence or presence of 10 μ M SB202190. Phase contrast images were recorded at 100x magnification. Similar results were obtained three times.



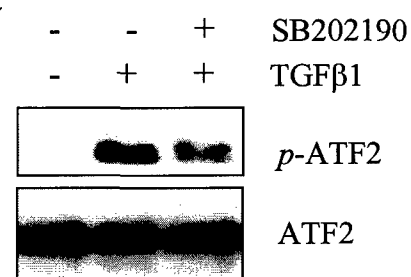
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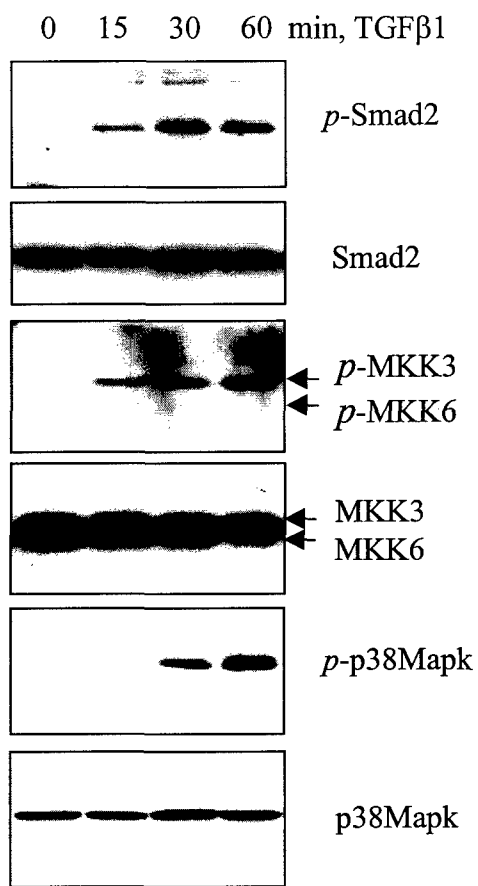
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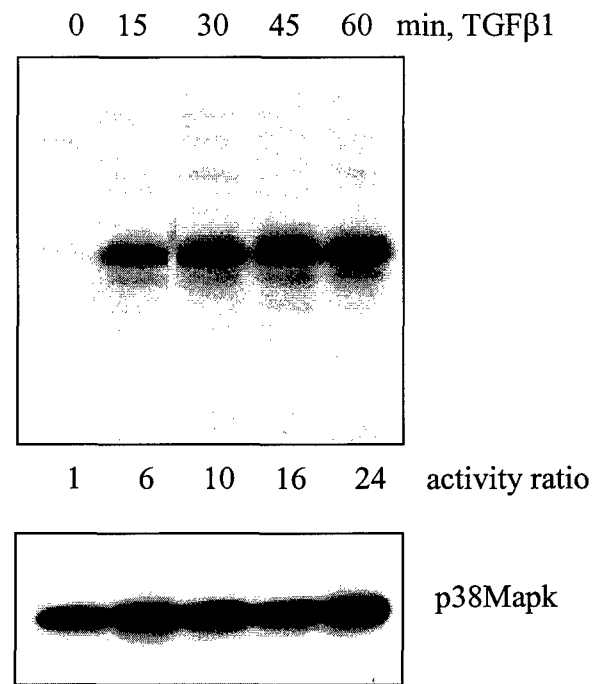
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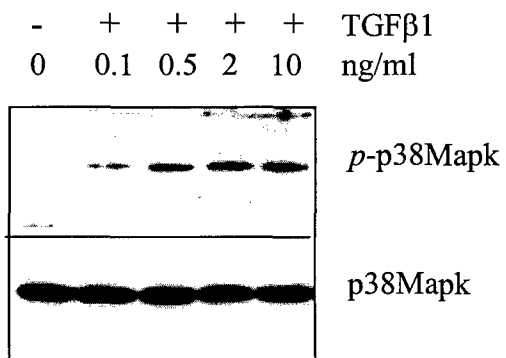
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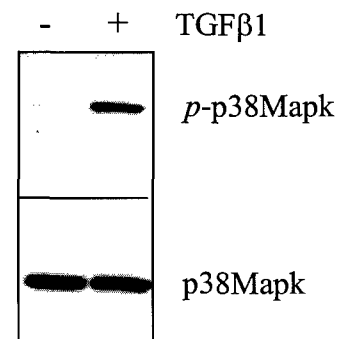
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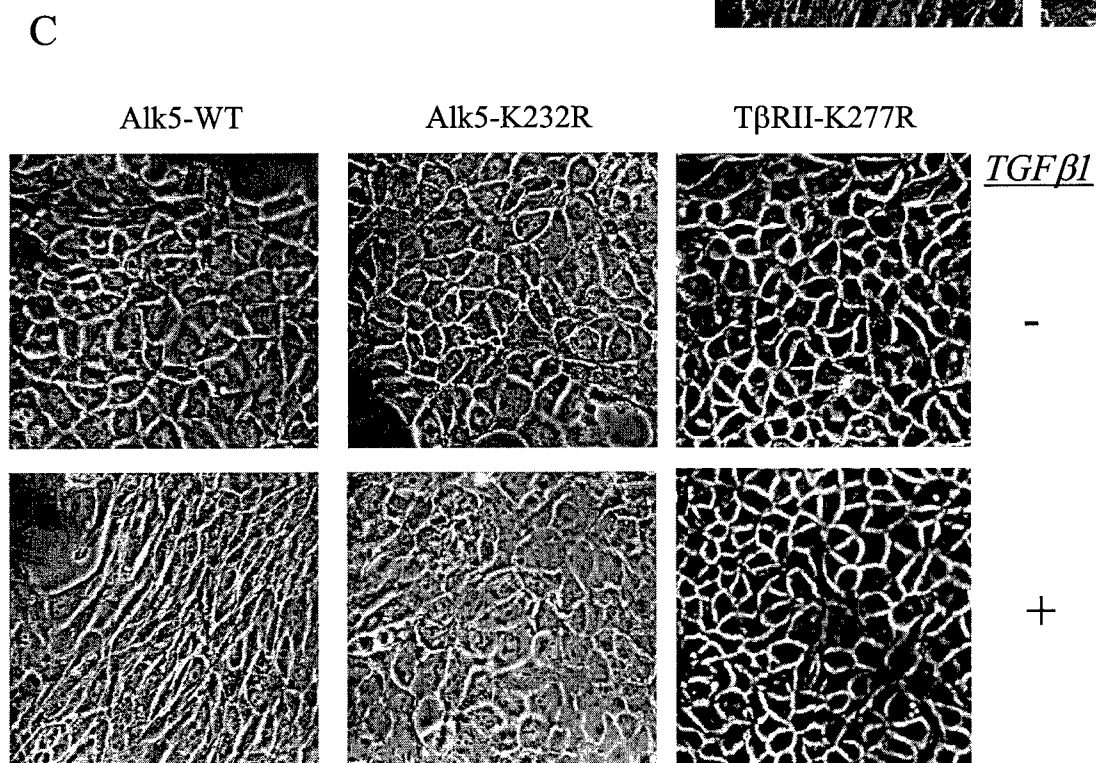
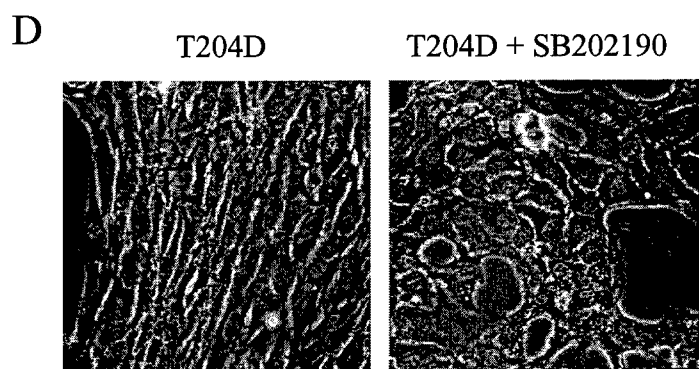
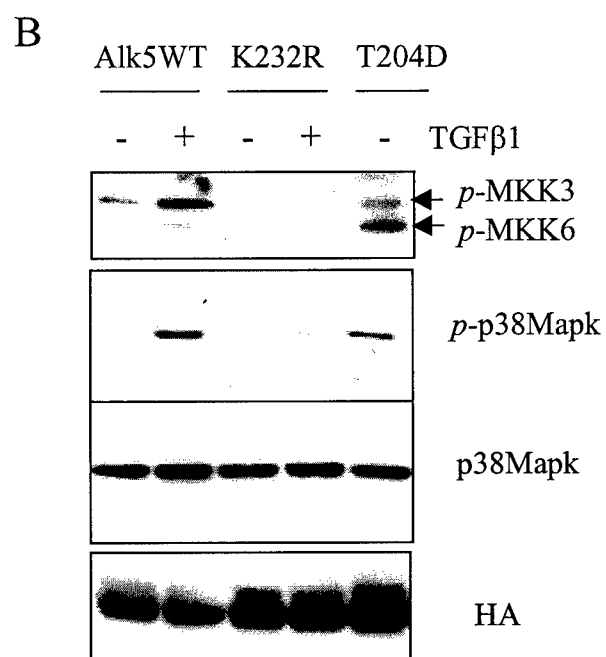
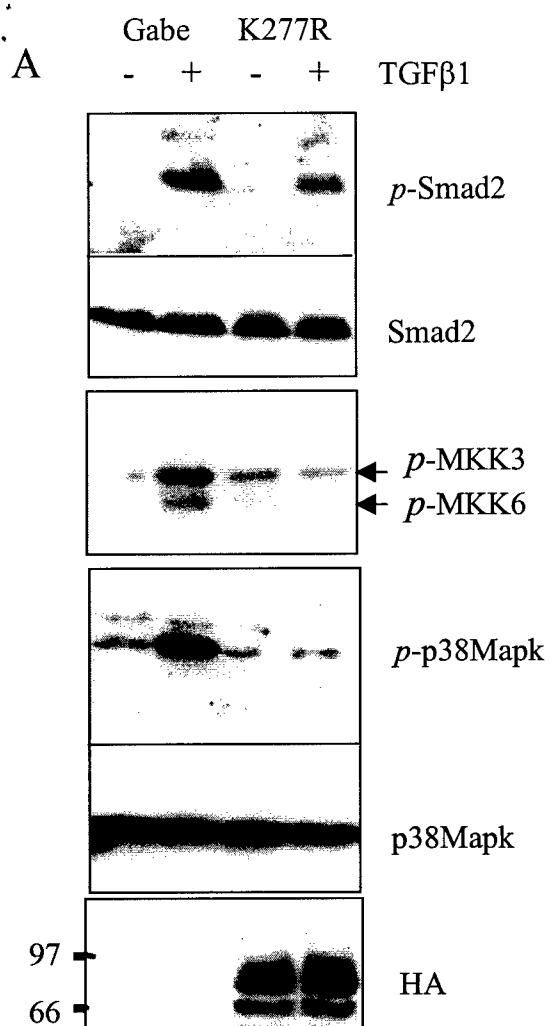


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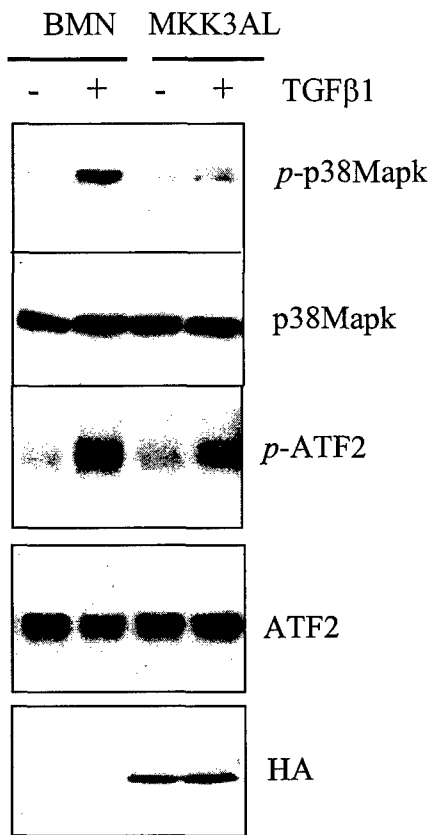


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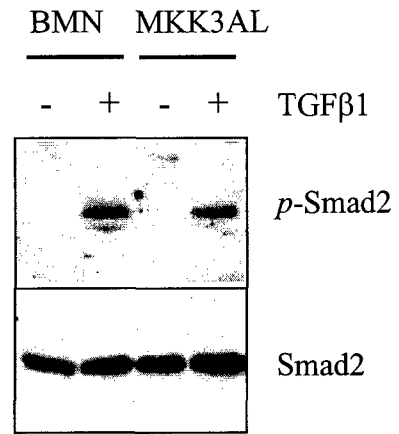




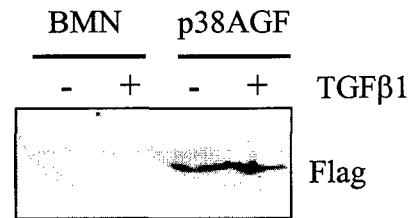
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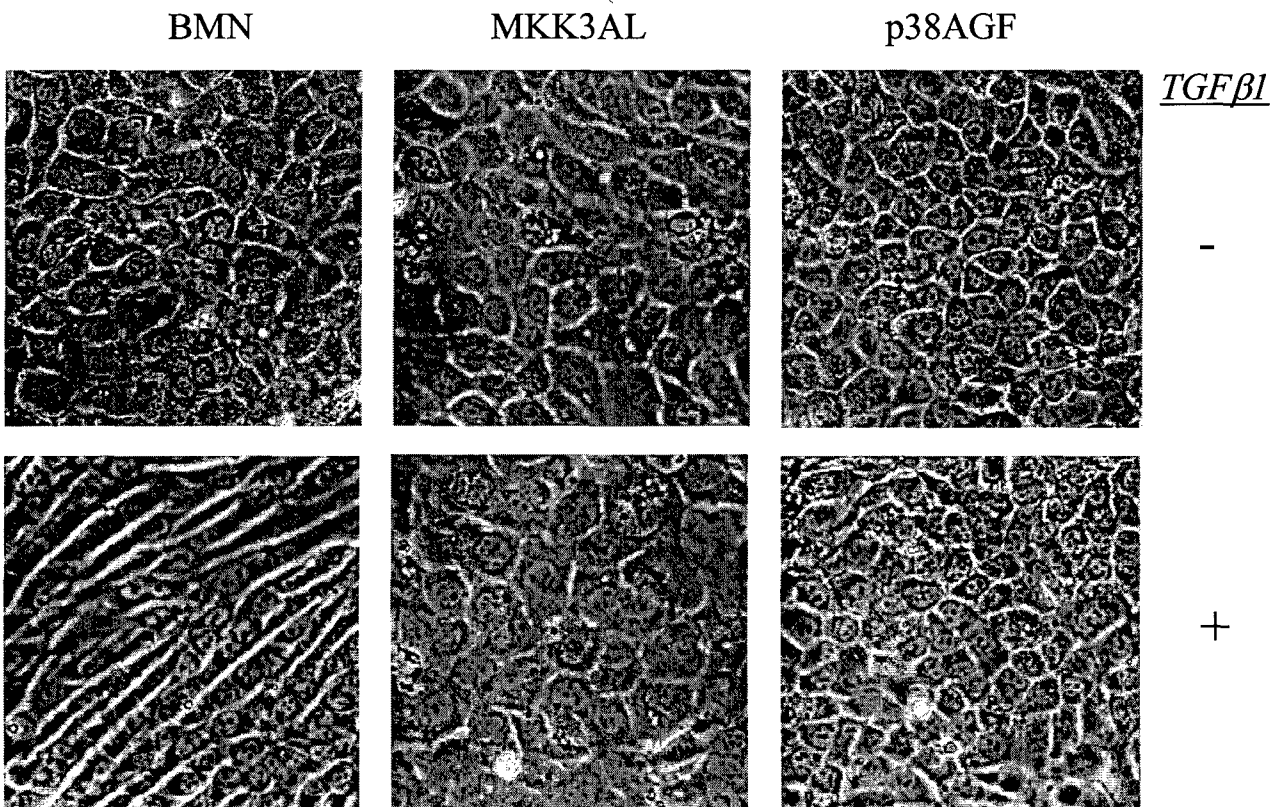
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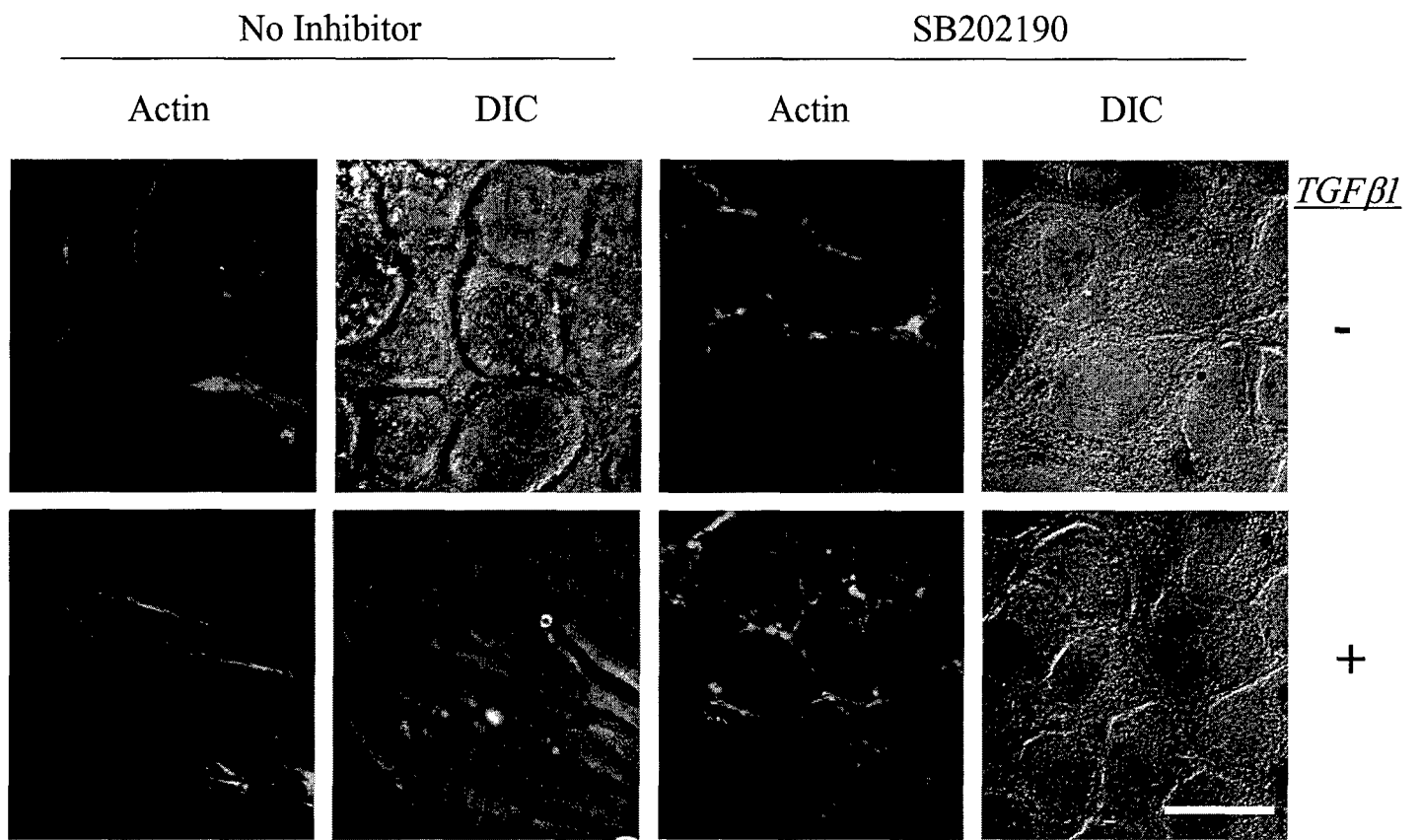
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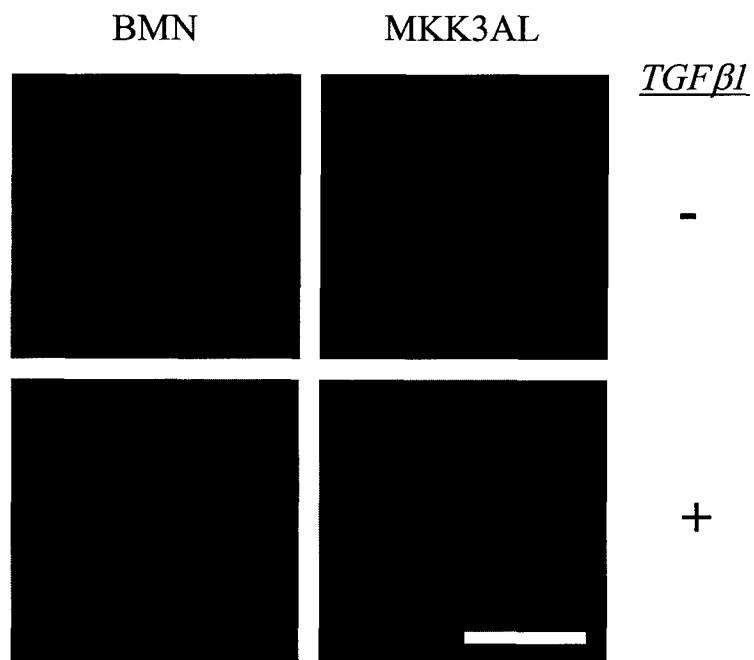
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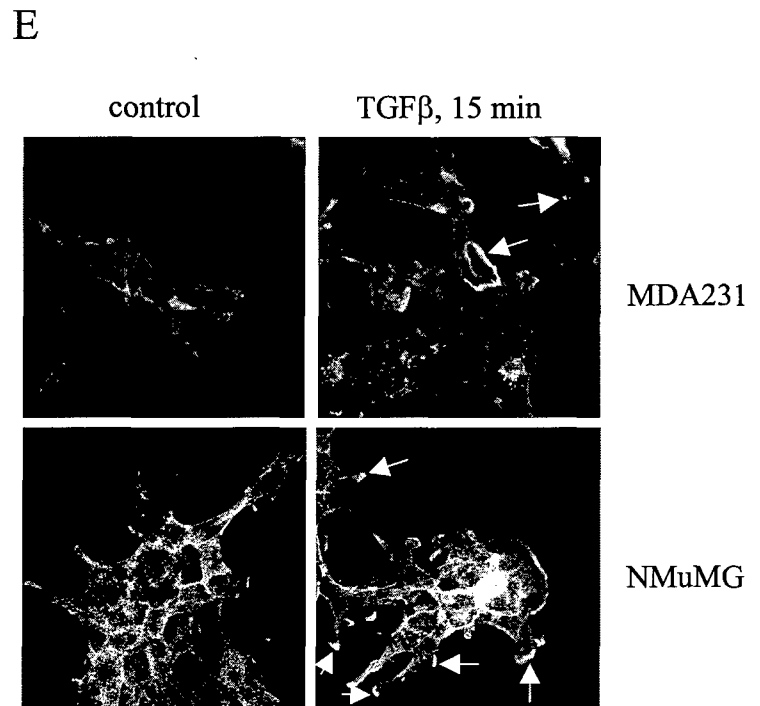
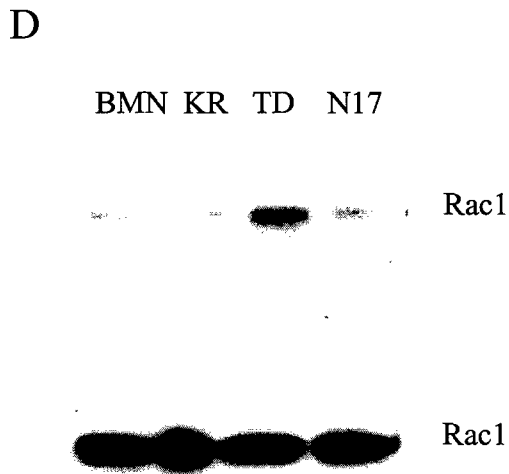
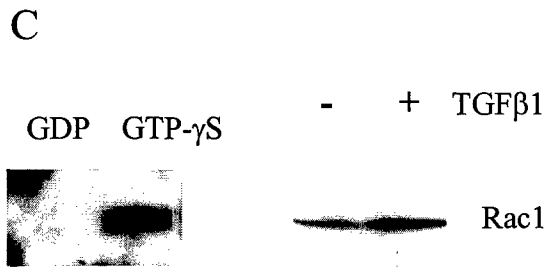
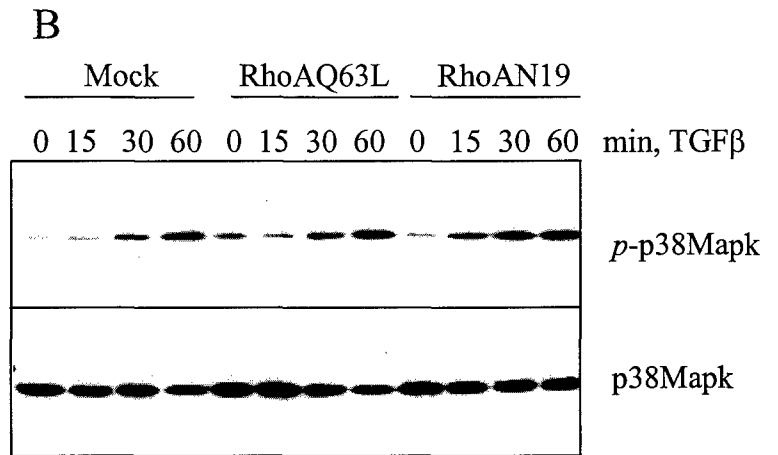
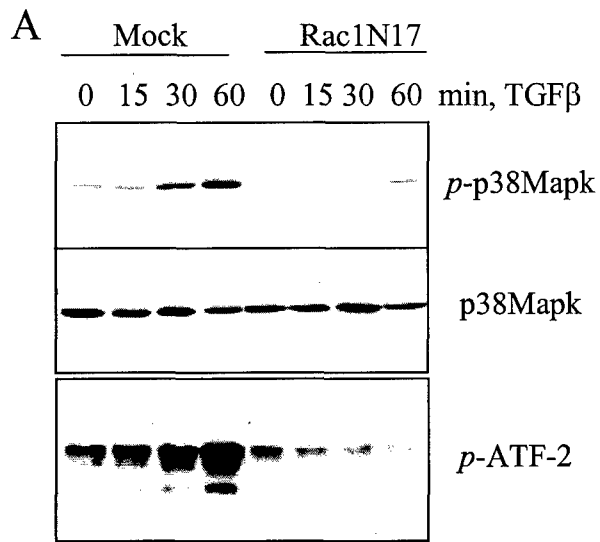


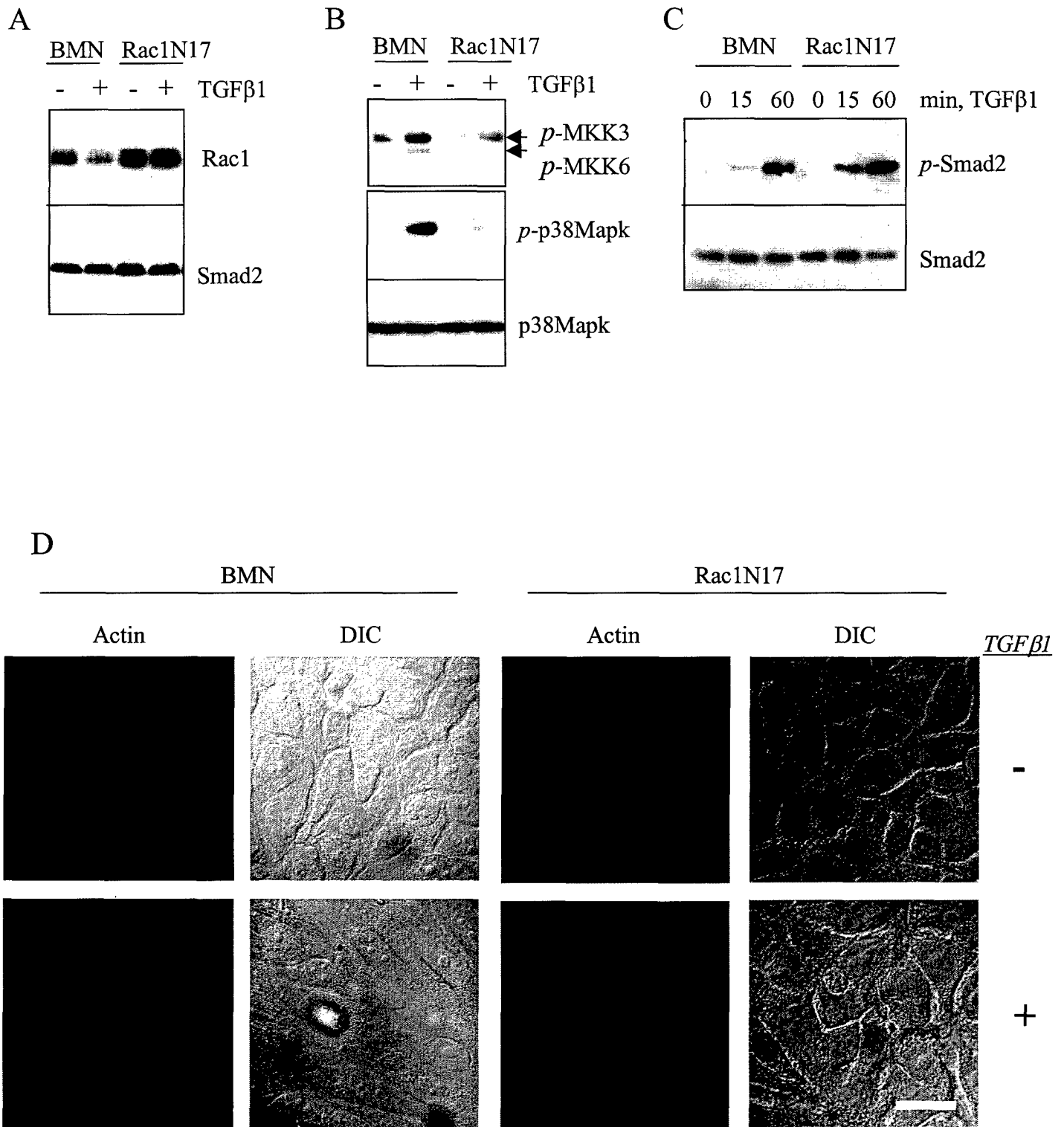
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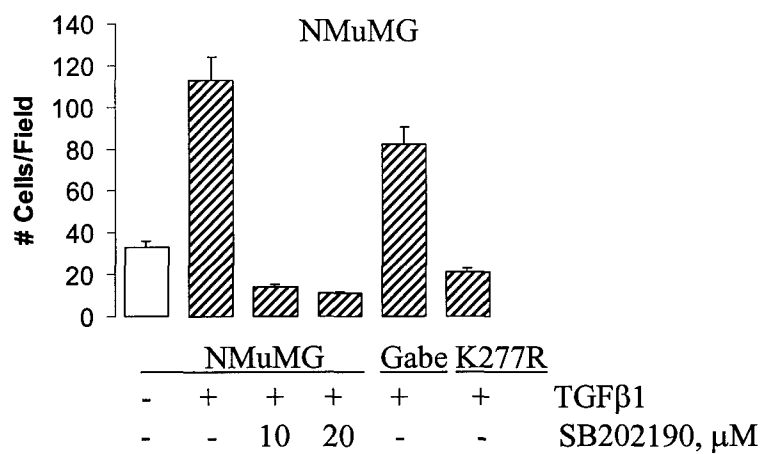
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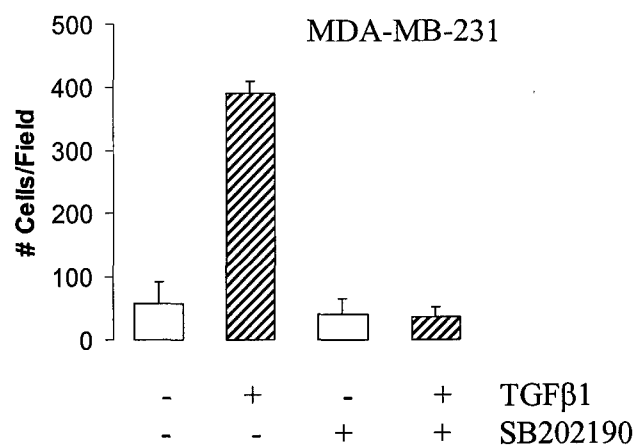




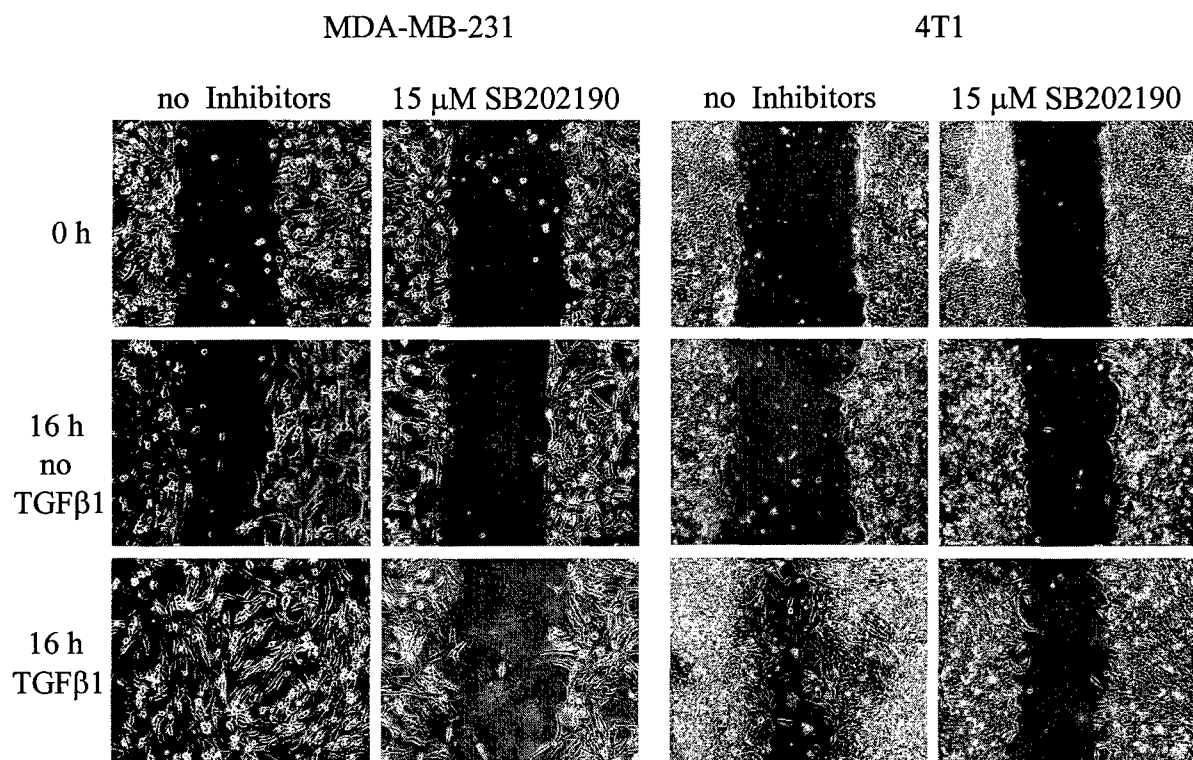
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Smad Signaling is not Sufficient for TGF β -mediated Motility in Human Cancer Cells

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Running title: TGF β signaling and tumor cell motility

SUMMARY

Transforming growth factor- β (TGF β) is a pleiotropic growth factor, which plays a critical role in modulating cell growth, differentiation and plasticity. There is increasing evidence that after cells lose their sensitivity to TGF β -mediated growth inhibition, autocrine TGF β signaling may potentially promote tumor cell motility and invasiveness. In order to understand the molecular mechanisms by which autocrine TGF β may selectively contribute to tumor cell motility, we have generated MDA-MB-231 breast cancer cells stably expressing a kinase-inactive T β RII (T β RII-K277R). Our data indicate that T β RII-K277R is expressed, can associate with the type I TGF β receptor, and block both Smad-dependent and -independent signaling pathways activated by TGF β . In addition, wound closure and transwell migration assays indicated that the basal migratory potential of T β RII-K277R expressing cells is impaired. The impaired motility of T β RII-K277R cells could be restored by reconstituting TGF β signaling with a constitutively active TGF β type I receptor (ALK5^{T204D}), but not by reconstituting Smad signaling with Smad2/4 or Smad3/4 expression. Likewise, re-expression of Smad4 in both the Smad4-defective MDA-MB-468 breast cancer cells and the SW480.7 colorectal cancer cells, did not promote tumor cell migration. These data suggest that Smad signaling alone is not sufficient for TGF β -mediated motility in human cancer cells.

INTRODUCTION

Transforming growth factor- β (TGF β)¹ is a pleiotropic polypeptide growth factor that is part of a superfamily of structurally related ligands which includes the TGF β s, activins, and bone morphogenetic proteins (BMPs) (1). TGF β ligands play a critical role in modulating cell growth, differentiation, plasticity and migration. They elicit their biological effects by binding to a heteromeric complex of transmembrane serine/threonine kinases, the type I and type II receptors. TGF β ligands can also bind to a transmembrane proteoglycan referred to as the type III receptor, which is thought to present ligand to the signaling type I and type II receptors. Following ligand binding to the type II receptor, the type I receptor is recruited to the complex. This allows the type II receptor, which is a constitutively active kinase, to transphosphorylate and thereby activate the type I receptor (2). Multiple pathways have now been implicated in mediating TGF β effects downstream of these receptors. These include the extracellular-regulated kinase (ERK) (3,4), c-Jun NH₂-terminal kinase (JNK) (5-7), p38 mitogen-activated protein kinase (MAPK) (8,9), and phosphatidylinositol-3 kinase (PI3K) pathways (10,11). Several small GTPases can also be activated by TGF β (12), and are involved in the activation of many of the above-mentioned signaling pathways. However, the Smad pathway was the first signaling pathway identified to mediate TGF β effects, and remains the best characterized of these (reviewed in (1)). Signal transduction through the Smad pathway involves phosphorylation of a set of intracellular signaling proteins termed receptor-regulated Smads (R-Smads) by the activated type I receptor. Once phosphorylated, R-Smads can associate with a common mediator Smad, Smad4, translocate to the nucleus, and regulate gene transcription. In addition to the R-Smads and the common mediator Smad, Smad4, there is a distinct, structurally related class of antagonistic Smads, Smad6 and Smad7, which inhibit TGF β family signals. Smad6 preferentially inhibits

BMP signaling by either competing with Smad4 for binding to R-Smads (13), or interfering with BMP receptor-mediated phosphorylation of Smads (14). Smad7 has been reported to inhibit both TGF β and BMP signaling by binding to activated type I receptors and interfering with their ability to phosphorylate R-Smads (15,16).

Although TGF β 1 was originally identified for its ability to cause reversible phenotypic transformation and anchorage independent growth of fibroblasts (17,18), it is now clear that TGF β can act both as a tumor suppressor and a tumor promoter. TGF β elicits most of its tumor suppressor activity by potently inhibiting the proliferation of most epithelial cells. It is thought that escape from the growth inhibitory effects of TGF β through dysregulated expression or mutational inactivation of various components of the TGF β signaling pathway can contribute to tumorigenesis (19-21). In addition, there is increasing evidence that after cells lose their sensitivity to TGF β -mediated growth inhibition, autocrine TGF β signaling may promote tumorigenesis. The importance of autocrine TGF β signaling in tumor progression has been highlighted by several studies which have shown that expression of a dominant negative type II TGF β receptor (dnT β RII) in various tumor cells can prevent the conversion of cells from an epithelial to a more invasive mesenchymal phenotype, delay tumor growth, and significantly reduce metastases (22-25). These data suggest that TGF β can act directly on tumor cells to promote tumor maintenance and progression. In addition to promoting epithelial to mesenchymal transformation of tumor cells, TGF β can stimulate the motility of many cell types *in vitro*, suggesting that TGF β production *in vivo* may enhance migration of tumor cells and thus contribute to tumor invasiveness and metastases. There is also evidence that TGF β can increase cellular motility without affecting proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways (26).

In order to understand the molecular mechanisms by which autocrine TGF β may selectively contribute to tumor cell motility, we have generated MDA-MB-231 breast cancer cells stably expressing a dnT β RII. MDA-MB-231 cells express TGF β receptors (27), secrete TGF β (28), and, although they are resistant to the growth inhibitory effects of TGF β (27), can respond to TGF β with an increase in spreading (29) and invasiveness (30). In addition, there is evidence that blocking TGF β signaling by administration of a neutralizing TGF β antibody can inhibit MDA-MB-231 cell tumorigenicity and metastases in nude mice (31). In this paper we show that expression of a dnT β RII in these cells, impairs their basal migratory potential. This impairment in motility can be restored by expression of a constitutively active type I TGF β receptor, but not by overexpression of Smad2/4 or Smad3/4, suggesting that Smad signaling alone is not sufficient for autocrine TGF β -mediated motility.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

The MDA-MB-231 and MDA-MB-468 breast cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD), and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The SW480.7 clone 15.13 (32) was a gift from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY), and was maintained in DMEM supplemented with 10% FCS, 0.3 mg/ml Zeocin, and 0.7 mg/ml G418. TGF β 1 and BMP2 were obtained from R&D Systems (Minneapolis, MN). Peter ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden) graciously provided the rabbit polyclonal sera directed against activin-like receptor kinases (ALKs) (33). Antibodies against the haemagglutinin (HA) epitope (Cat #sc-7392), the type II

TGF β receptor (Cat #sc-220), Smad4 (Cat #sc-7966) and p38 MAPK (Cat #sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to fibronectin (Cat #F14420), and Smad2/3 (Cat #S66220) were from Transduction Laboratories (San Diego, CA). The C-terminal phospho-Smad2 antibody (Cat #06-829) was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). The C-terminal phospho-Smad1 antibody (Cat #9511) and the phospho-p38 MAPK antibody (Cat #9211S) were from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies to actin (Cat #A-4700) and the FLAG epitope (Cat #F3165) as well as the polyclonal antibody to α -catenin (Cat #C2081) were obtained from Sigma-Aldrich (St Louis, MO). Phalloidin-Texas Red and Hoechst 3342 were from Molecular Probes (Eugene, Oregon).

Generation of Stable Cell Lines

To generate MDA-MB-231 cells stably expressing a dnT β RII, we obtained a construct encoding a kinase inactive T β RII in which the lysine at position 277 has been mutated to arginine (pGABE-T β RII-K277R) (23) from Martin Oft (UCSF, San Francisco, CA). Lysine 277 corresponds to an invariant lysine found in the ATP-binding site of subdomain II in all protein kinases, and even its substitution with arginine results in loss of kinase activity (34). The pGABE vector is a modified version of the commonly used retroviral vector pBABE in which the puromycin cassette has been replaced by enhanced green fluorescent protein (GFP). In this construct, T β RII-K277R is HA tagged, and its expression is driven by the viral long terminal repeat, while expression of GFP is driven by the SV40 promoter. MDA-MB-231 cells were transfected with the control pGABE vector or the pGABE-T β RII-K277R vector utilizing LipofectAMINE reagent (Gibco BRL, Rockville, MD), according to manufacturer's instructions.

Following transfection, cells expressing GFP were sorted by flow cytometry. Clones were then isolated by sorting individual cells from the > 95% positive GFP pool.

Affinity Labeling of Cells with 125 I-TGF β 1 and Immunoprecipitation of HA-tagged T β RII-K277R

125 I-TGF β 1 was obtained from NEN Life Science Products, Inc. (Boston, MA). Near confluent MDA-MB-231 cells as well as clones and pools stably expressing GFP alone (GABE) or GFP and T β RII-K277R (dnT β RII) in 12-well plates were washed three times over 30 min with 500 μ l of ice-cold 0.1% bovine serum albumin (BSA)/D-PBS containing Ca^{+2} and Mg^{+2} . The cells were then affinity labeled with 100 pM 125 I-TGF β 1 as previously described (35), with slight modifications. Briefly, after a 3 h incubation with 100 pM 125 I-TGF β 1 at 4°C, the cells were washed with 500 μ l ice-cold D-PBS and the ligand-receptor complexes were cross-linked with 400 μ l of 1 mM bis(sulfosuccinimidyl)suberate (BS3) (Pierce, Rockford, IL) for 10 min on ice. The cross-linking reaction was stopped with the addition of 100 μ l of 500 mM glycine. Cells were washed twice with 500 μ l D-PBS, and solubilized with 125 μ l of 20 mM Tris buffer pH 7.4 containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, and 2 μ g/ml soybean trypsin inhibitor. Solubilized material was centrifuged for 10 min at 4°C to pellet cell debris. The supernatants were transferred to one-fifth volume of 5x electrophoresis sample buffer, boiled and vortexed. All samples were analyzed using 3-12% SDS-PAGE and visualized by autoradiography. For immunoprecipitation experiments, the radiolabeled cell lysate from a T75 flask was centrifuged at 5000 x g, the supernatant was split into eight equal aliquots, and incubated with antibodies directed against ALKs 1, 2, and 5, the type II TGF β receptor, or HA overnight at 4°C. Aliquots

of radiolabeled cell lysates incubated with normal rabbit serum or no antibody were used as controls.

Immunoblot Analysis

Cells were washed twice with ice cold D-PBS and lysed with 50 mM Tris/150 mM NaCl buffer containing 1% NP-40, 0.25% deoxycholate, 1 mM EDTA, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin, and 2 µg/ml soybean trypsin inhibitor. Protein content was quantitated utilizing the BCA Protein Assay Reagent (Pierce, Rockford, IL). Protein extracts were separated by 7.5% or 10% SDS-PAGE and transferred to nitrocellulose membranes at 100 volts for 2 h. Membranes were blocked with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20 [v/v]) for 1 h at room temperature (RT) and incubated with primary antibodies diluted in TBS-T plus 2.5% nonfat dry milk over night at 4°C. The membranes were then washed four times for 10 min with TBS-T, incubated with HRP-conjugated secondary antibodies for 1 h at RT and rewashed four times for 10 min with TBS-T. Immunoreactive bands were visualized by chemiluminescence (Pierce).

Immunofluorescence

Cells grown on glass cover-slips (22x22 mm) in 35 mm wells were washed twice with D-PBS, fixed with 4% paraformaldehyde in D-PBS for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 3% nonfat dry milk in D-PBS for 60 min, all at RT. Cells were then incubated with primary mouse monoclonal antibodies diluted in 1% nonfat dry milk/D-PBS for 1 h at RT, washed three times with D-PBS, followed by incubation with a CY3-conjugated

anti-mouse antibody diluted 1:500 in 1% nonfat dry milk/PBS for an additional hour at RT. In some experiments, cell nuclei were stained with 1 $\mu\text{g}/\text{ml}$ Hoechst for 10 min at RT. After three 10 min washes with D-PBS, cover-slips were mounted onto 25x75-mm microslides using AquaPolyMount (Polysciences Inc., Warrington, PA). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

Transcription Reporter Assays

Cells were transiently transfected with 1 μg per 35 mm dish of the Smad-dependent heterologous promoter reporter construct p(CAGA)₁₂-Luciferase (36) (provided by J.-M. Gauthier, Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) along with 0.01 μg per 35 mm dish pCMV-Renilla using FuGENE6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. The following day, cells were split into 24-well plates, and approximately 45 h post transfection, cells were either left unstimulated or were stimulated with 40 pM TGF β 1 for 16-20 h. All cells were then washed with D-PBS and lysed. Firefly and *Renilla reniformis* luciferase activities were measured using Promega's Dual Luciferase Reporter Assay System according to the manufacturer's protocol. Luciferase activity was normalized utilizing the ratio of Firefly to *Renilla reniformis* luciferase activity and presented as fold induction. All assays were done in triplicate wells and each experiment was repeated at least twice.

Wound Closure and Transwell Motility Assays

For wound closure assays, confluent cell monolayers were wounded by manually scraping the cells with a pipet tip. Following wounding, wound size was verified with an ocular

ruler to ensure that all wounds were the same width. The cell culture medium was then replaced with fresh medium and wound closure was monitored by microscopy at various times.

Transwell motility assays were performed utilizing 5- μ m pore, 6.5 mm polycarbonate Transwell filters (Corning Costar Corp., Cambridge, MA). For these assays, single cell suspensions were seeded in serum-free media containing 0.1% BSA onto the upper surface of the filters and allowed to migrate towards various concentrations of FCS. After a 16-20 h incubation period, cells on the upper surface of the filter were wiped off with a cotton swab, and the cells that had migrated to the underside of the filter were fixed, stained with 0.5% crystal violet, and counted by brightfield microscopy at 200x in five random fields.

Adenoviral Expression of ALKs and Smads

The adenoviral construct encoding FLAG-tagged Smad4 (37) was obtained from Dr. Harold Moses (Vanderbilt University, Nashville, TN). All other adenoviral constructs encoding FLAG-tagged Smads or HA-tagged constitutively active mutants of the TGF β (ALK5^{T204D}), activin (ALK2^{Q207D}) and BMP (ALK3^{Q233D}) type I receptors (38) were generously provided by Dr. Kohei Miyazono (Japanese Foundation for Cancer Research, Tokyo, Japan). Stocks of recombinant viruses for each of these constructs were generated in 293 cells and titered utilizing the Takara Assay (Takara Biomedicals, Tokyo, Japan). Cells were then infected with these or a control β -galactosidase adenovirus (β -GAL) at a multiplicity of infection (moi) which resulted in >90% cell infection (approximately 15 pfu/cell or less). The efficiency of infection was evaluated by *in situ* staining of cells for β -galactosidase activity 48 h following infection.

RESULTS

TβRII-K277R is Expressed in MDA-MB-231 Cells

In order to abrogate TGFβ signaling in MDA-MB-231 cells, a construct encoding GFP and a kinase inactive TβRII receptor was transfected into the cells. Expression of the kinase inactive TβRII-K277R mutant was verified by affinity labeling cell surface receptors with ¹²⁵I-TGFβ1 (Fig. 1A). Since TβRII-K277R has an intact extracellular domain, it can still bind TGFβ, and should therefore co-migrate with endogenous TβRII, as both are the same size. Cell surface labeling of parental cells resulted in the labeling of three proteins corresponding to the endogenous type I, II, and III TGFβ receptors. There was little or no change in the amount of receptor labeling observed in the control cells expressing GFP alone (GABE 15 Pool) compared to parental cells. However, in the pool expressing TβRII-K277R (dnTβRII 15 Pool), there was a significant increase in the amount of labeled type II receptor, suggesting that the exogenous receptor was expressed. Individual clones obtained from each of these pools expressing either GFP alone (G15-5, -6) or GFP and TβRII-K277R (dn15-2, -3, -5, -11) displayed a similar pattern of labeling.

In order to confirm that this increase in the labeling of TβRII was indeed due to expression of the HA-tagged TβRII-K277R, extracts from affinity labeled cells were immunoprecipitated with an HA antibody. As shown in Fig. 1B, the HA antibody immunoprecipitated a labeled type II receptor in the pool and clones expressing TβRII-K277R, but not in the control pool or clones expressing GFP alone, confirming transgene expression. The type I TGFβ receptor appeared to co-immunoprecipitate with TβRII-K277R in these experiments. This was confirmed in subsequent co-immunoprecipitation experiments (see Fig. 2).

TβRII-K277R is Functional in MDA-MB-231 Cells

Having ascertained that TβRII-K277R was expressed, we then wished to determine whether it was functional. Co-immunoprecipitation experiments were performed in order to determine whether TβRII-K277R could associate with the type I TGFβ receptor (TβRI). These experiments revealed that when affinity labeled cells expressing TβRII-K277R were immunoprecipitated with an HA antibody, a labeled protein the size of a type I receptor co-immunoprecipitated with TβRII-K277R (Fig. 1B and Fig. 2, right panel – lane 8). We confirmed that this was TβRI by immunoprecipitating similarly labeled cells with various TGFβ superfamily type I receptor antibodies, including ALK1, 2, and 5. Only the ALK5 (TβRI), but not the ALK1 or 2 antibodies precipitated the cross-linked type I receptor (Fig. 2). While the TβRII antibody co-immunoprecipitated ALK5 efficiently, the ALK5 antibody, co-immunoprecipitated TβRII only weakly (Fig. 2 lane 5, both panels). In the control GABE 15 Pool, the HA antibody failed to immunoprecipitate any proteins, as expected. Immunoprecipitations with a TβRII antibody were carried out in both pools as a positive control, and both resulted in the co-immunoprecipitation of TβRI (Fig. 2, lane 7 in both panels). These data indicate that TβRII-K277R associates with TβRI.

In order to determine whether TβRII-K277R could also prevent TGFβ signaling, we next examined its effect on the ability of TβRI to phosphorylate Smad2. Immunoblot analysis of TGFβ1-treated cell lysates using a phospho-specific Smad2 antibody revealed that while TGFβ1 could induce phosphorylation of Smad2 in both GABE clones (G15-5 and G15-6), its ability to do so in the TβRII-K277R clones (dn15-2, -3, -5, -11) was impaired (Fig. 3A). This impairment was not due to a decrease in total Smad2 protein, as reprobing with an antibody directed against

the unphosphorylated form of Smad2 did not reveal any significant change in total Smad2 protein. We next examined the effect of T β RII-K277R expression on TGF β -induced Smad translocation to the nucleus by immunofluorescence (Fig. 3B). In the GABE clone, in the absence of TGF β , Smad2 staining was relatively diffuse, but upon TGF β 1 treatment for 60 min, Smad2 staining became concentrated in the nucleus. Nuclear localization of Smad2 was confirmed by staining the same cells with Hoechst. In contrast, in the T β RII-K277R clones, there was little or no change in Smad2 staining following TGF β 1 treatment, suggesting that TGF β -mediated translocation of Smad2 to the nucleus is impaired by T β RII-K277R expression. We then examined the effect of T β RII-K277R expression on TGF β 1-induced transcription. A reporter construct containing twelve Smad binding elements repeated in tandem, p(CAGA)₁₂-Luciferase, was transiently transfected into the GABE and T β RII-K277R clones along with pCMV-Renilla. Normalized luciferase activity indicated that TGF β 1 could induce transcription of both reporter constructs in the GABE clones, but its ability to do so in the T β RII-K277R clones was impaired (Fig. 3C). Similar results were obtained using the p(3TP)-Luciferase reporter (data not shown).

In order to determine whether signaling pathways other than the Smad pathway were also affected by expression of T β RII-K277R, we examined fibronectin expression, which has been reported to be induced by TGF β in a JNK-dependent, but Smad4-independent manner (6). We chose to perform these experiments in our pools as the results obtained in these cells are representative of those obtained in the clones (compare phospho-Smad2 blots in Fig. 3A and D). Following TGF β stimulation for 24 h, we observed an increase in fibronectin expression in the GABE pool, but this induction was significantly decreased in the pool expressing T β RII-K277R, as was the basal level of fibronectin expression (Fig. 3D). We were unable to detect any

induction of phosphorylation of JNK or AKT in response to TGF β in our GABE pools (data not shown). However, we did observe an increase in phosphorylation of p38 MAPK following TGF β stimulation for 60 min, and this induction of phosphorylation was attenuated in the pool expressing T β RII-K277R (Fig. 3D).

T β RII-K277R Impairs the Motility of MDA-MB-231 Cells

Next we examined the effect of T β RII-K277R expression on the motility of MDA-MB-231 cells in a wound closure assay. In the GABE clones, cells migrated into the wounded area and closed the wound within 24 h, while in the T β RII-K277R clones the wound remained open at 24 h (Fig. 4A). This difference in motility did not appear to be due to an effect on proliferation because when the experiment was performed in the presence of mitomycin C, a compound that inhibits cell division, the same results were obtained (data not shown). Thus, expression of T β RII-K277R in MDA-MB-231 cells appears to impair their motility, independent of changes in proliferation. As an alternative measure of cell motility, we also examined the effect of T β RII-K277R on the ability of cells to migrate in a transwell assay system. We observed a three to four fold reduction in the ability of cells expressing T β RII-K277R to migrate towards FCS, as compared with control cells expressing GFP alone (Fig. 4B).

The Impaired Motility of T β RII-K277R Cells is TGF β Type I Receptor Specific

Since the impaired motility of T β RII-K277R cells was observed in the absence of exogenous TGF β stimulation, we wished to determine whether this impairment was TGF β specific. Because T β RII-K277R acts in a dominant negative manner by virtue of its ability to associate with, and prevent the activation of, the endogenous type I receptor, we chose to restore

TGF β signaling in T β RII-K277R cells by expressing a constitutively active mutant of T β RI. Mutation of threonine 204 in ALK5 (T β RI) to aspartic acid leads to constitutive activation of the type I receptor kinase, allowing it to induce signals in the absence of ligands or type II receptors (39). Likewise, mutation of corresponding threonine and glutamine residues in the activin (40) and BMP (41) type I receptors to aspartic acid also leads to constitutive activation of these kinases. To test for TGF β specificity, cells expressing T β RII-K277R were infected with adenoviruses encoding HA-tagged constitutively active mutants of the TGF β (ALK5^{TD}), activin (ALK2^{QD}) and BMP (ALK3^{QD}) type I receptors (38). Uninfected cells or cells infected with a β -galactosidase adenovirus at the same moi were used as controls. The efficiency of infection was >90% as evaluated by *in situ* staining of cells for β -galactosidase activity 48 h following infection (data not shown). At this time, expression of the mutant type I receptors was confirmed by immunoblot analysis utilizing an HA antibody (Fig. 5A), and their effect on motility was assessed in wound closure assays. Motility was only restored in cells expressing ALK5^{TD} (Fig. 5C). Although ALK2^{QD} and ALK3^{QD} were expressed and functional, as evidenced by their ability to induce Smad1 phosphorylation (Fig. 5B), they failed to restore motility in cells expressing T β RII-K277R (Fig. 5C). These results suggest that the impaired motility of T β RII-K277R cells is T β RI (ALK5) specific.

Restoration of Smad Signaling does not Rescue the Impaired Motility of T β RII-K277R Cells

Although some biological responses mediated by TGF β are clearly Smad dependent (42), it is unclear whether TGF β -mediated motility requires Smad signaling. We reasoned that if Smads are required for TGF β -mediated motility, expression of the inhibitory Smad, Smad7, in the parental or control GABE cells should impair their motility. However, the levels of Smad7

expression required to block TGF β signaling resulted in cell death (data not shown). Therefore, it was not possible to address whether TGF β -mediated motility requires Smad signaling utilizing this approach. Instead, we chose to overexpress the TGF β R-Smads, Smad2 or Smad3 along with Smad4 in order to determine whether reconstitution of Smad signaling in cells expressing T β R β II-K277R could restore motility. Cells were infected with adenoviruses encoding FLAG-tagged Smad2 and Smad4 or FLAG-tagged Smad3 and Smad4, and exogenous Smad expression was confirmed by immunoblot analysis utilizing an anti-FLAG antibody (Fig. 6A). The ability of Smad2/4 and Smad3/4 to activate Smad-dependent signaling was examined utilizing the Smad-dependent transcription reporter construct, p(CAGA)₁₂-Luciferase. Expression of Smad3/4 resulted in a marked increase in basal transcription (Fig. 6B). Stimulation with TGF β 1 did not cause any further increase in transcription, suggesting that Smad signaling was maximally activated. Despite this, Smad3/4 failed to restore motility in the cells expressing T β R β II-K277R (Fig. 6C, bottom panel), and had no effect on the motility of control cells expressing GFP alone (Fig. 6C, top panel). Activation of basal transcription was not as marked with Smad2/4, as expected, since Smad2 itself cannot bind DNA (43). However, despite nearly 100-fold induction of transcription following infection at the maximally tolerated moi, this combination also failed to restore motility in the cells expressing T β R β II-K277R (Fig. 6C, bottom panel), and had no effect on the motility of control cells expressing GFP alone (Fig. 6C, top panel). These results indicate that reconstitution of Smad signaling alone is not sufficient to restore autocrine TGF β -mediated motility in cells expressing T β R β II-K277R, nor is it sufficient to enhance the motility of control MDA-MB-231 cells. This suggests that Smad signaling may not be required for autocrine TGF β -mediated motility or that additional TGF β -regulated signaling pathways cooperate with Smads to elicit this effect.

Re-expression of Smad4 in Smad4-defective Cancer Cells does not Enhance Motility

In order to determine if Smads are required for cancer cell migration, we examined whether activation of TGF β signaling could promote motility in the absence of Smad signaling utilizing the MDA-MB-468 breast cancer cells which lack endogenous Smad4 (44). Smad4 and ALK5^{TD} were expressed, either alone or in combination, by adenoviral transduction, and their effects on the motility of MDA-MB-468 cells was examined in wound closure assays. Expression of HA-tagged ALK5^{TD} and Flag-tagged Smad4 was confirmed by immunoblot analysis (Fig. 7A), and their ability to activate Smad-dependent signaling was examined in transcription reporter assays utilizing the Smad-dependent p(CAGA)₁₂-Luciferase reporter construct (Fig. 7B). As expected, in the absence of Smad4 (Uninfected, β GAL, ALK5^{TD} alone), TGF β 1 was unable to stimulate transcription in these cells. However, upon re-expression of Smad4 a marked increase in both TGF β 1-mediated and ALK5^{TD}-mediated transcription was observed, indicating that both Smad4 and ALK5^{TD} were indeed functional in these cells. Despite this, neither Smad4 nor ALK5^{TD} had any effect on cell motility, whether they were expressed alone or in combination (Fig. 7C). The fact that ALK5^{TD} could not promote motility, even when Smad4 was co-expressed with it, suggests that MDA-MB-468 cells are not responsive to the pro-migratory effects of TGF β .

In order to determine whether TGF β could induce migration in the absence of Smad signaling in other cells, the Smad4 defective SW480.7 colorectal cells, conditionally expressing Smad4 via an ecdysone-inducible system (32), were utilized. Cells were stimulated with increasing concentrations of TGF β 1 in the absence or presence of 3 μ M ponasterone to induce Smad4 expression. Smad4 expression in ponasterone-treated cells was confirmed by

immunoblot analysis (Fig. 8A), and its effect on TGF β -mediated motility was examined in wound closure assays. Again, as in the MDA-MB-468 cells, these cells failed to respond to TGF β both in the absence and presence of Smad4 (Fig. 8B). Taken together, these data indicate that Smad signaling alone is not sufficient to promote migration of cancer cells.

Discussion

In this study, abrogation of autocrine TGF β signaling in MDA-MB-231 breast cancer cells resulted in an impairment in their basal migratory potential, which could not be restored by reconstituting Smad signaling. These results suggest that Smad signaling alone is not sufficient for autocrine TGF β -mediated motility. However, whether Smad signaling is required, in addition to other pathways activated by TGF β , to mediate the migration of cancer cells remains an open question as TGF β failed to promote migration in the Smad4 defective MDA-MB-468 and SW480.7 cells whether Smad4 was expressed or not.

Although the role of Smads in cancer cell migration has, to the best of our knowledge, never been examined, previous studies in non-transformed cells have generated conflicting data on the requirement of Smad signaling in cell migration (45-48). In one study, expression of a dominant negative Smad3 mutant in non-transformed murine mammary cells had no effect on TGF β -mediated motility even though it blocked the anti-mitogenic effect of TGF β (45). This suggests that Smad3 may not be required for this response, or that residual Smad3 signaling, not blocked by expression of the dominant negative Smad3 mutant, may still be sufficient to mediate motility. This would be consistent with the idea that different biological responses require different thresholds of TGF β signaling. Thus, complete abrogation of Smad3 signaling might be

required to observe an impairment in TGF β -mediated motility whereas partial blockade of Smad function might be sufficient to subvert the antiproliferative effects of TGF β .

Complete abrogation of signaling is difficult to achieve with dominant negative approaches, and expression of antagonistic Smad7 resulted in cell death in our hands. This is in accord with previous studies which have reported that overexpression of Smad7 can sensitize various cell types to cell death (49). Therefore, we chose to address this question by activating Smad signaling rather than blocking it. Having ascertained that the impaired motility of T β RII-K277R cells was indeed T β RI specific, we overexpressed Smad2 or Smad3 along with Smad4 in these cells to determine whether autocrine TGF β -mediated motility was Smad dependent. Despite their ability to activate Smad-dependent transcription, neither Smad combination restored the impaired motility of the T β RII-K277R cells. These data suggest that in breast cancer cells, autocrine TGF β signaling mediates motility in a Smad-independent manner or that alternative pathways, in addition to the Smad signaling pathway are required for these effects. If autocrine TGF β -mediated motility were Smad-independent, this would imply that non-transformed cells and transformed cells utilize different mechanisms to elicit similar biological responses as others have reported that Smad3-null monocytes and keratinocytes exhibit significantly reduced migration to TGF β 1 in transwell motility assays (46). Moreover, Smad3 appears to be required for TGF β -mediated monocyte chemotaxis *in vivo*, as mice lacking the Smad3 gene display a blunted monocyte chemotactic response following cutaneous wounding (46). Studies in *Drosophila* also suggest that Smads may be required for cell migration as mutations in Mad, the *Drosophila* receptor-activated Smad, impair migration of the epidermis during dorsal closure (47). Finally, recent studies in endothelial cells have indicated that TGF β acting through ALK1 stimulates migration in a Smad-dependent manner, while TGF β acting

through ALK5 inhibits cell migration in a Smad-dependent manner (48). Taken together, these studies highlight the importance of Smads in TGF β -regulated migration of non-transformed cells.

A lack of requirement for Smad signaling in TGF β -mediated cancer cell migration would be consistent with previous studies which have shown that TGF β can increase cellular motility of prostate cancer cells without affecting proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways (26). Likewise, expression of Smad7 in pancreatic cancer cells has been shown to abrogate the anti-proliferative effects of TGF β , but enhance matrix associated transcriptional responses, highlighting a dissociation between the matrix and anti-proliferative effects induced by TGF β (50). If the biological effects of TGF β that can contribute to tumor progression were Smad independent, it might be possible to selectively disrupt those pathways, while ensuring that the tumor suppressive, Smad-dependent pathways are maintained. Recent studies aimed at identifying the mechanisms by which TGF β elicits an epithelial to mesenchymal transition in mammary cells have indicated that the PI3K, RhoA and p38 MAPK pathways are involved in this process (9,11,45,51). However, whether Smad signaling is required for the epithelial to mesenchymal transition induced by TGF β is unclear. In one study, adenoviral expression of low levels of constitutively active ALK5 induced epithelial to mesenchymal transition only if Smad2/4 or Smad3/4 were co-expressed (52). On the other hand, others have reported that inhibition of Smad signaling either by overexpression of Smad7 or dominant negative Smad3 did not affect the transdifferentiation, arguing against the involvement of Smads in epithelial to mesenchymal transition (45). It will be of interest to determine whether such phenotypic changes require Smad signaling as a

dissociation between the pathways required for the tumor suppressive versus the tumor promoting effects of TGF β , could potentially allow for selective therapeutic interventions.

The fact that T β RII-K277R was expressed at levels high enough to block the basal migratory potential of MDA-MB-231 cells is important in light of the fact that different biological responses induced by TGF β seem to require different thresholds of signaling. For example, expression of a dnT β RII in squamous carcinoma cells blocked the growth inhibitory effects of TGF β , but failed to block its ability to induce epithelial to mesenchymal transition (24). Likewise, expression of a dnT β RII in 4T1 murine breast cancer cells impaired TGF β -mediated transcription, but failed to block motility (25). Therefore, the molecular mechanisms by which autocrine TGF β may selectively contribute to tumor progression could not be fully addressed in these models. In addition, different signaling pathways activated by TGF β also appear to require different thresholds of signaling as previous studies have shown that expression of a dnT β RII in normal murine mammary cells impaired TGF β -mediated activation of Smad-dependent signaling, but not TGF β -mediated activation of p38 MAPK (9). Since we have achieved T β RII-K277R expression levels high enough to block both Smad and non-Smad pathways in MDA-MB-231 cells, the model we have generated will prove useful in dissecting the signaling pathways required for the diverse effects elicited by TGF β in cancer.

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Footnotes:

¹The abbreviations used are: TGF β , transforming growth factor- β ; BMP, bone morphogenetic protein; ERK, extracellular-regulated kinase; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3 kinase; R-Smad, receptor-regulated Smad; dnT β RII, dominant negative TGF β receptor type II; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ALK, activin-like receptor kinase; HA, haemagglutinin; GFP, enhanced green fluorescent protein; RT, room temperature; β -GAL, β -galactosidase; moi, multiplicity of infection; T β RI, TGF β receptor type I.

FIGURE LEGENDS

Fig. 1. **T β RII-K277R is expressed in MDA-MB-231 Cells.**

MDA-MB-231 parental cells as well as clones and pools stably expressing GFP alone (G15-5, -6 clones and G15 Pool) or GFP and T β RII-K277R (dn15-2, -3, -5, -11 clones and dn15 Pool) were affinity labeled with 100 pM 125 I-TGF β 1 and cross-linked with BS³. Labeled ligand-receptor complexes were resolved by SDS-PAGE using a 3-12% gradient gel and visualized by autoradiography (A), or lysed and incubated with a mouse monoclonal anti-HA antibody for immunoprecipitation of HA-tagged T β RII-K277R (B). Immunoprecipitates were resolved by SDS-PAGE using a 7.5% polyacrylamide gel and visualized by autoradiography. Affinity labeled, but non-immunoprecipitated G15-5 cells were loaded as a reference (NIP).

Fig. 2. **T β RII-K277R can associate with T β RI.**

MDA-MB-231 pools expressing GFP alone (GABE 15 Pool, left panel) or GFP and T β RII-K277R (dnT β RII 15 Pool, right panel), were affinity labeled with 100 pM 125 I-TGF β 1, cross-linked with BS³, lysed and incubated with NRS (normal rabbit serum), polyclonal rabbit antisera directed against various type I TGF β superfamily receptors (ALK1, 2 & 5), the type II TGF β receptor (T β RII), or HA, as indicated. Immunoprecipitates were resolved by SDS-PAGE using a 3-12% polyacrylamide gel and visualized by autoradiography. Affinity labeled, but non-immunoprecipitated cells were loaded as a reference (NIP).

Fig. 3. Expression of T β RII-K277R impairs TGF β signaling.

(A) Near confluent clones expressing GFP alone (G15-5 and G15-6) or GFP and T β RII-K277R (dn15-2, -3, and -11) were incubated overnight under serum-free conditions, stimulated with 80 pM TGF β 1 for the times indicated, washed, and lysed. Protein extracts (50 μ g/lane) were separated by 10% SDS-PAGE followed by immunoblot analysis for phospho-Smad2 (*p*-Smad2) and total Smad2/3. **(B)** Cells were grown on glass cover-slips for 48 h, serum starved for 16 h followed by treatment with 40 pM TGF β 1 for 1 h. Cells were then prepared for indirect immunofluorescence staining of Smad2, followed by nuclear staining with Hoechst. **(C)** Cells were transiently transfected with p(CAGA)₁₂-Luciferase along with pCMV-Renilla. The following day, cells were split to 6 wells of a 24-well plate, treated with 40 pM TGF β 1 for 16 h, washed, and lysed. Firefly and *Renilla* luciferase activities were measured using Promega's Dual Luciferase Reporter Assay System. Fold induction of luciferase activity (Y axis) is based on the ratio of firefly to *Renilla* luciferase activities. Each data point represents the mean \pm SD of three wells. **(D)** Near confluent pools expressing GFP alone (G15) or GFP and T β RII-K277R (dn 15) were incubated overnight under serum-free conditions, stimulated with 80 pM TGF β 1 for the times indicated, and prepared for immunoblot analysis as in **(A)**. The fibronectin blot (Fn) was probed with an antibody directed against α -catenin as a loading control, while the phospho-Smad2 (*p*-Smad2) and phospho-p38 MAPK (*p*-p38 MAPK) blots were probed with antibodies directed against the unphosphorylated forms of the respective proteins to verify equal loading.

Fig. 4. Expression of T β RII-K277R impairs motility.

(A) Confluent cell monolayers of clones expressing GFP alone (G15-5 and G15-6) or GFP and T β RII-K277R (dn15-2, -3, and -11) were wounded with a pipet tip. Following wounding, cell culture medium was replaced with fresh medium and wound closure was monitored by microscopy at the times indicated. (B) Single cell suspensions of pools expressing GFP alone (G15 Pool) or GFP and T β RII-K277R (dn15 Pool) in serum-free media containing 0.1% BSA were seeded onto 5 μ M polycarbonate transwell filters and allowed to migrate towards increasing concentrations of FCS, as indicated. After 20 h, cells on the underside of the filters were fixed, stained, and counted. The results are represented quantitatively in the bar graph below the representative filter micrographs. Each data point represents the mean \pm SD of two wells.

Fig. 5. Expression of ALK5^{TD}/T β RI restores motility in T β RII-K277R cells.

Clone dn15-2 was infected with adenoviruses encoding HA-tagged constitutively active mutants of the activin (ALK2^{QD}), BMP (ALK3^{QD}) or TGF β (ALK5^{TD}) type I receptors at an moi of 15. Uninfected cells, and cells infected with a β -galactosidase (β GAL) adenovirus at a similar moi were used as controls. Approximately 48 h following infection, ALK expression (A) and function (B) were verified by immunoblot analysis utilizing an anti-HA or a phospho-specific Smad1 antibody, as indicated. The blots were also probed with an actin antibody in order to verify equal loading. The effect of ALK expression on wound closure was monitored by microscopy at the times indicated (C).

Fig. 6. Expression of Smad2/4 or Smad3/4 does not restore motility in T β RII-K277R cells.

Cells were infected with both FLAG-tagged Smad2 and Smad4 (S2/4) or FLAG-tagged Smad3 and Smad4 (S3/4) encoding adenoviruses at an moi of 3 for Smad2/3 and an moi of 15 for Smad4. Uninfected cells (Uninf.), and cells infected with a β -galactosidase (β GAL) adenovirus were used as controls. Approximately 48 h following infection, Smad expression was verified by immunoblot analysis utilizing an anti-FLAG antibody (**A**). The ability of Smads to restore TGF β signaling in dn15-2 was evaluated in transcription reporter assays utilizing the TGF β responsive transcription reporter p(CAGA)₁₂-Luciferase (**B**). The effect of Smad expression on wound closure in the control G15 Pool (top panel) and dn15-2 clone (bottom panel) was monitored by microscopy at the times indicated (**C**).

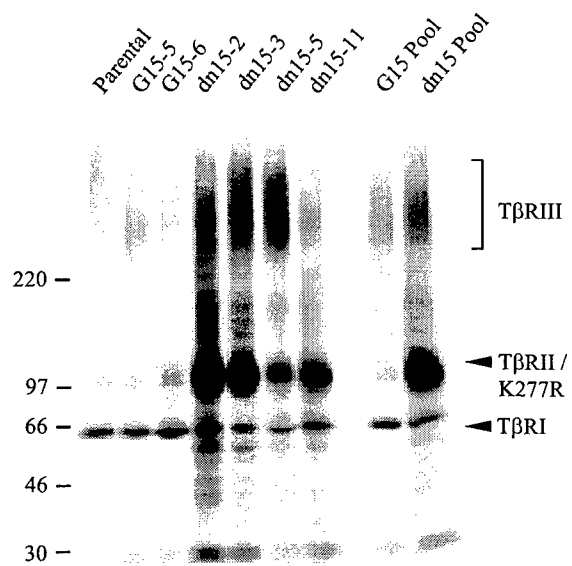
Fig. 7. Effect of ALK5^{TD} on the motility of MDA-MB-468 cells +/- Smad4.

Cells were infected with adenoviruses encoding FLAG-tagged Smad4 (S4), HA-tagged ALK5^{TD} (A5) or both (A5+S4) at an moi of 15. Uninfected cells (Uninf.), and cells infected with a β -galactosidase (β GAL) adenovirus were used as controls. Approximately 48 h following infection, exogenous Smad4 and ALK5^{TD} expression was verified by immunoblot analysis utilizing anti-FLAG and anti-HA antibodies, respectively. The blots were also probed with an actin antibody in order to verify equal loading (**A**). The ability of Smad4 and ALK5^{TD} to activate Smad-dependent signaling was evaluated in transcriptional reporter assays utilizing the Smad-dependent p(CAGA)₁₂-Luciferase reporter construct (**B**), and their effect on motility was examined in wound closure assays (**C**).

Fig. 8. Effect of exogenous TGF β 1-stimulation on the motility of SW480.7 cells +/- Smad4.

Cells in 6-well plates were either left untreated (left panel) or treated with 3 μ M ponasterone (right panel) for 40 h in order to induce Smad4 expression. Cells were then wounded, washed, and incubated with serum-free media in the presence of 0, 4, 20, or 100 pM TGF β 1 for 24 h. Wound closure was monitored by microscopy at the times indicated **(B)**. At the conclusion of the wound closure experiment, cells were lysed, and Smad4 expression was examined by immunoblot analysis utilizing a monoclonal antibody directed against Smad4 **(A)**. The blots were also probed with an actin antibody in order to verify equal loading. Ponasterone was maintained in the culture medium of selected wells throughout the experiment.

A.



B.

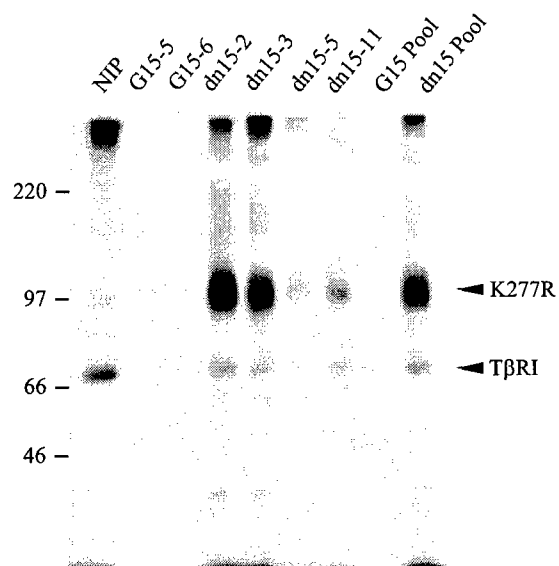


Fig.1

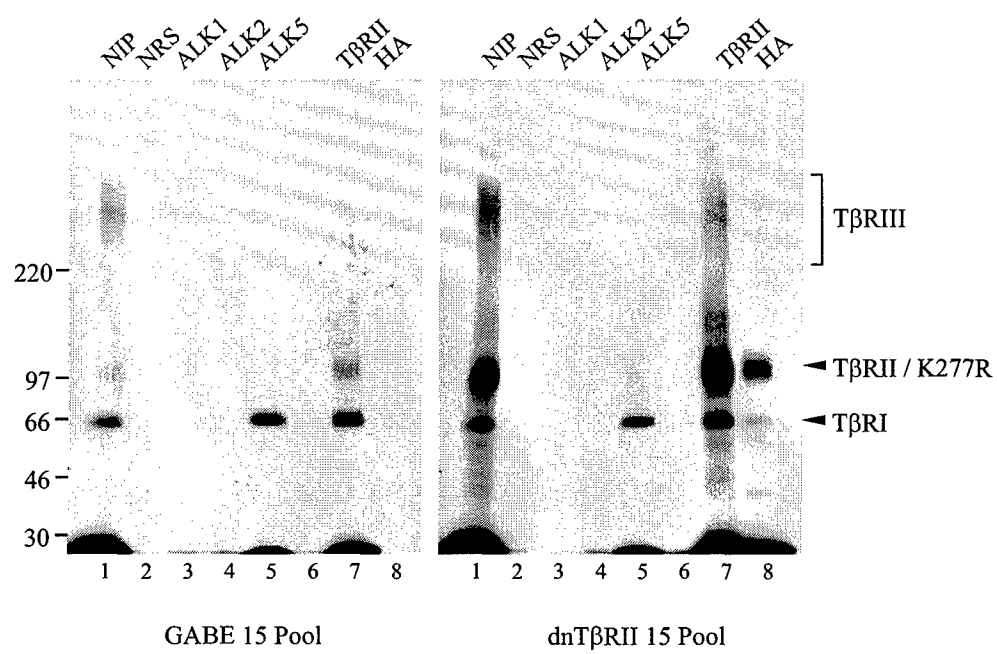


Fig.2

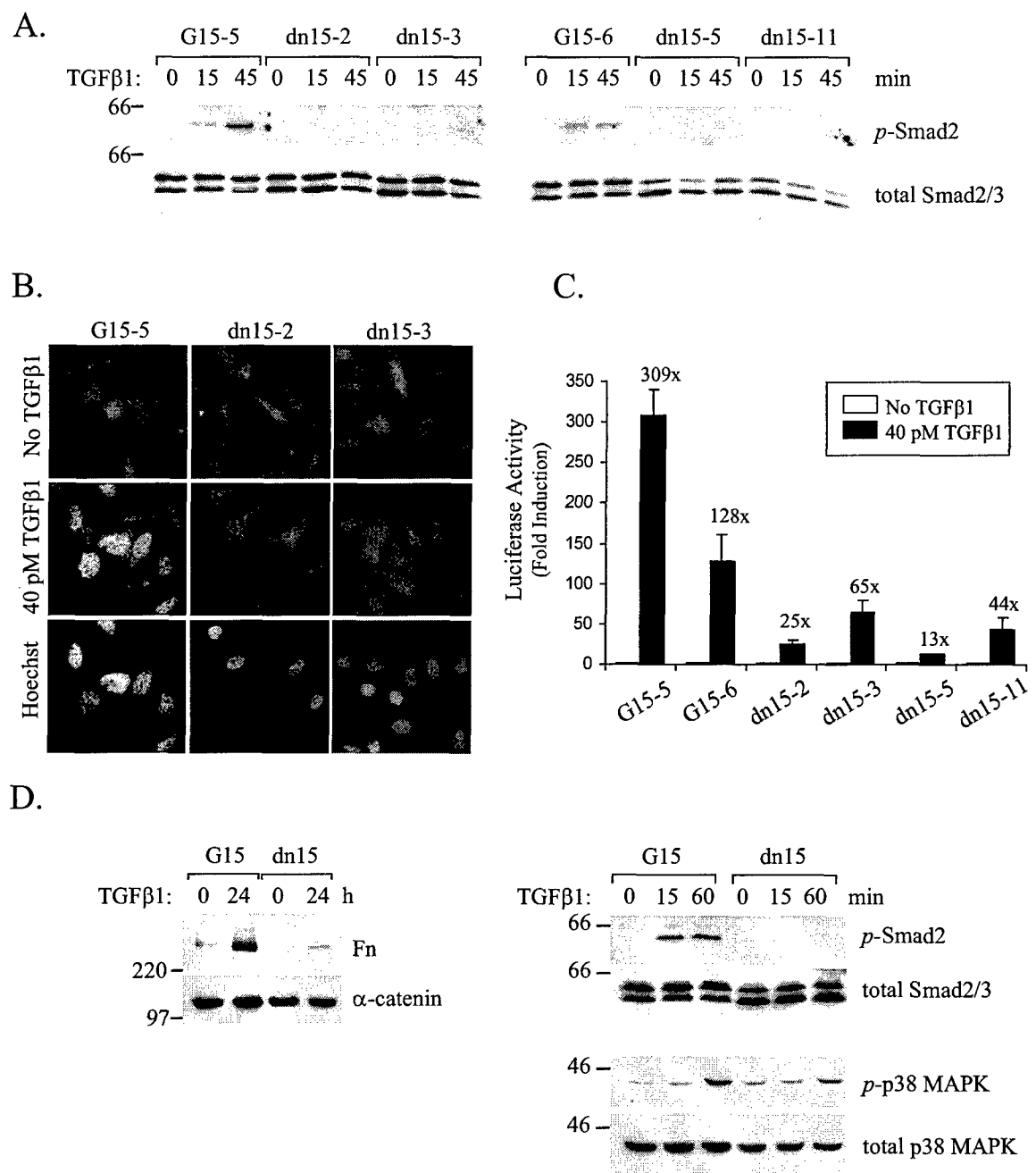
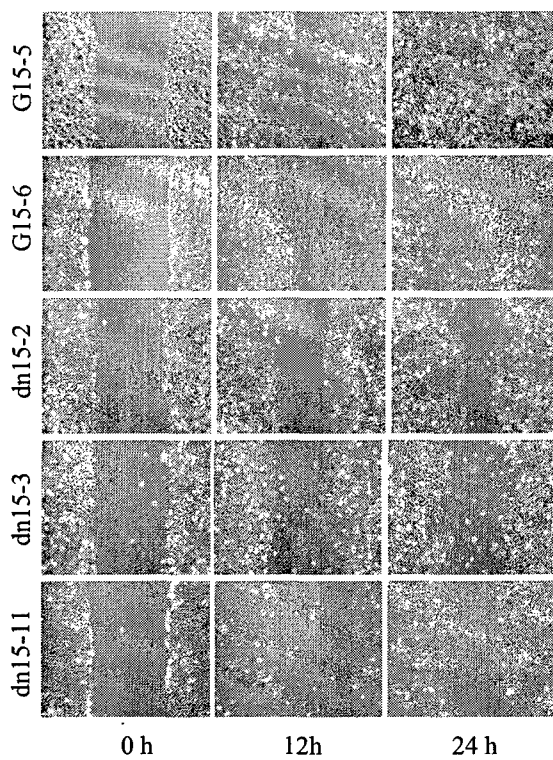


Fig. 3

A.



B.

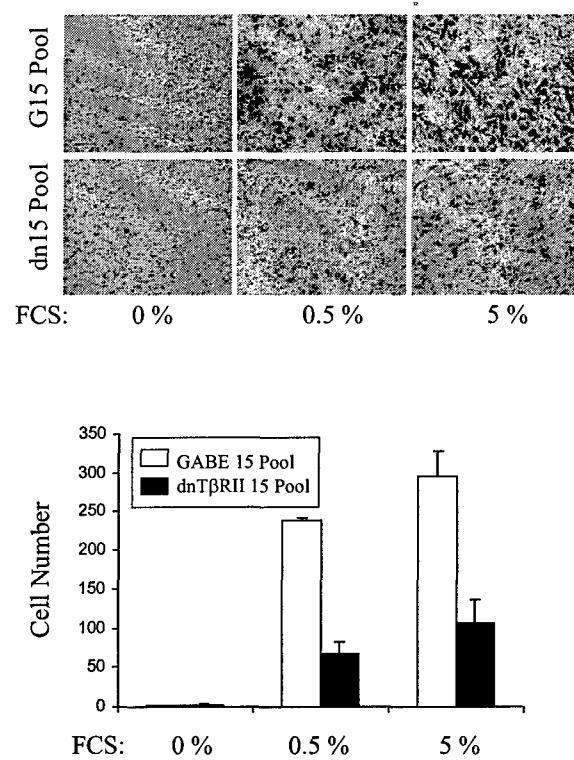


Fig. 4

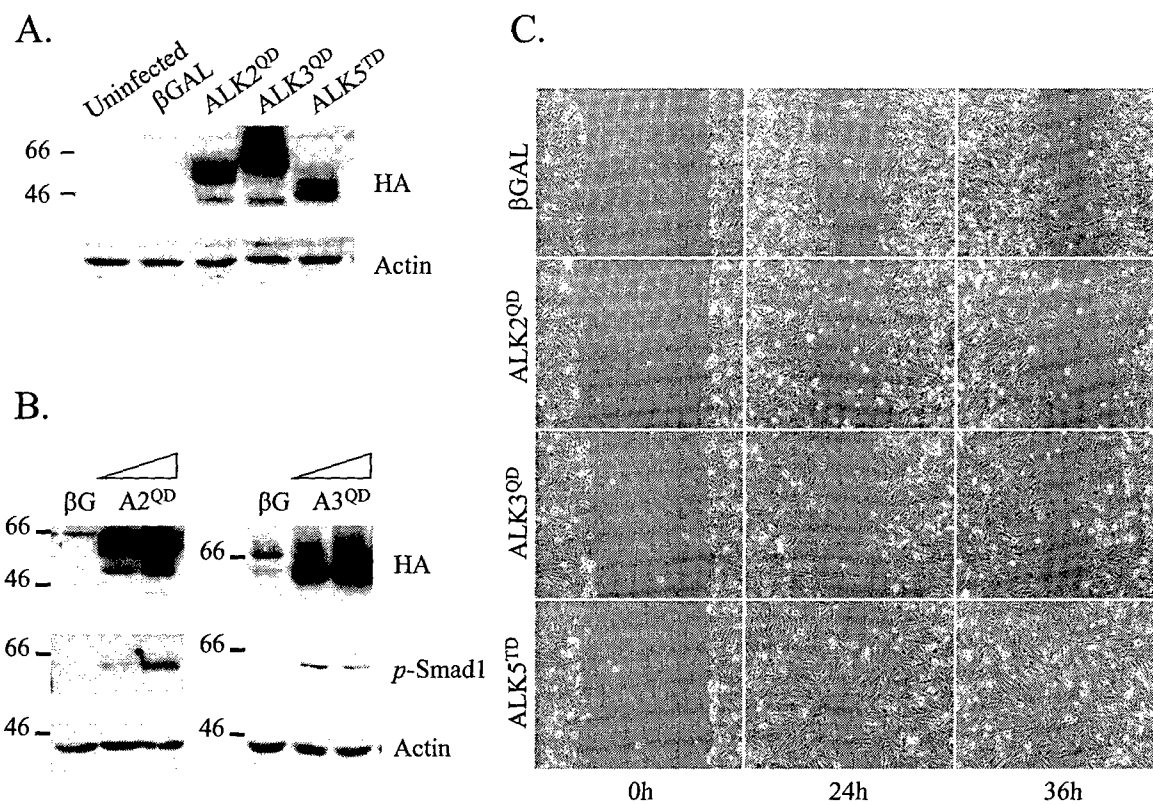


Fig. 5

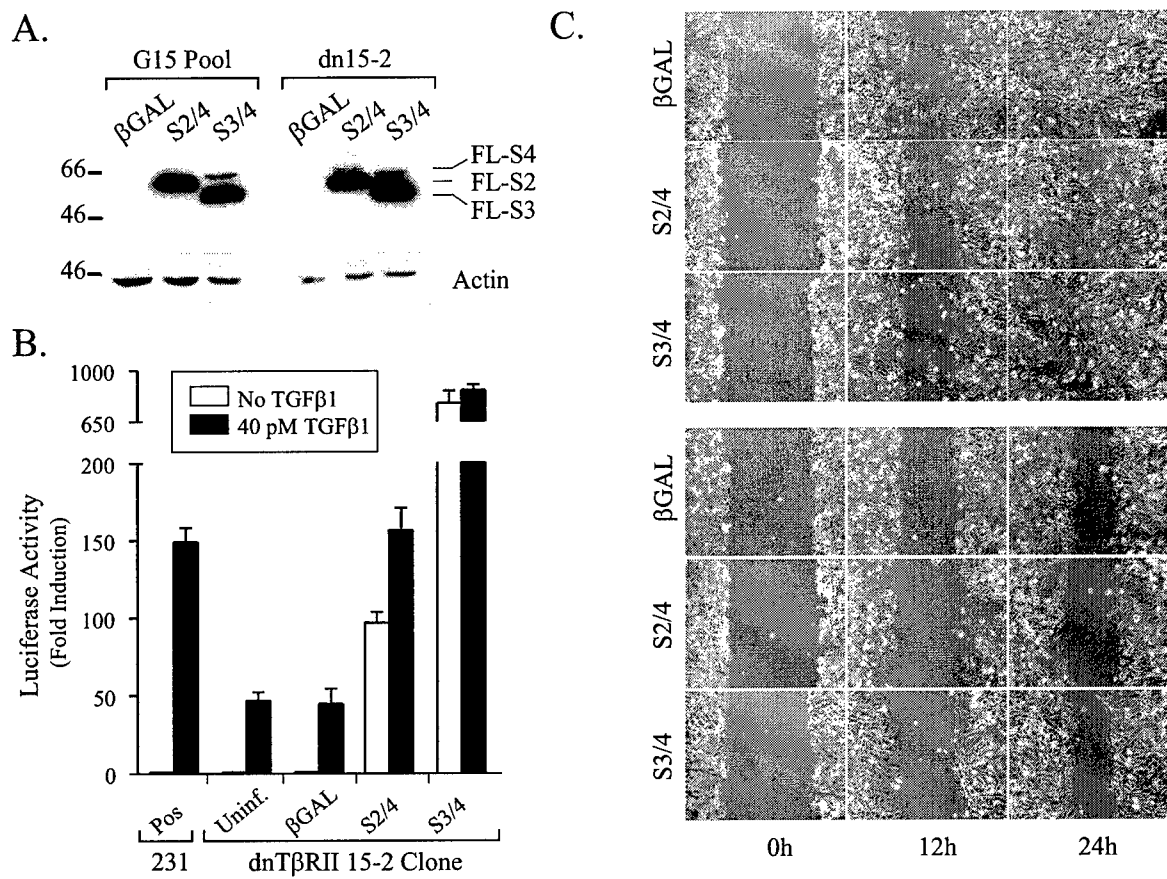


Fig. 6

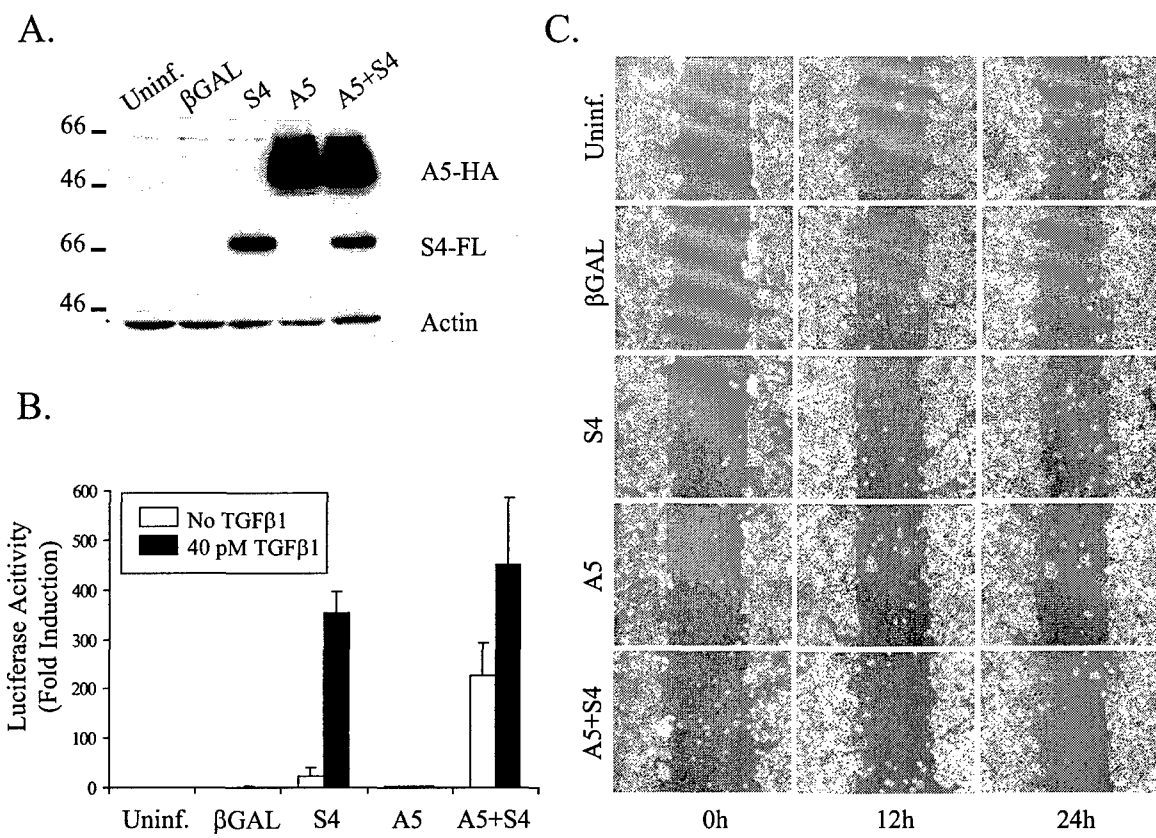
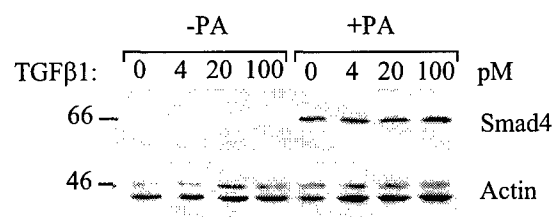


Fig. 7

A.



B.

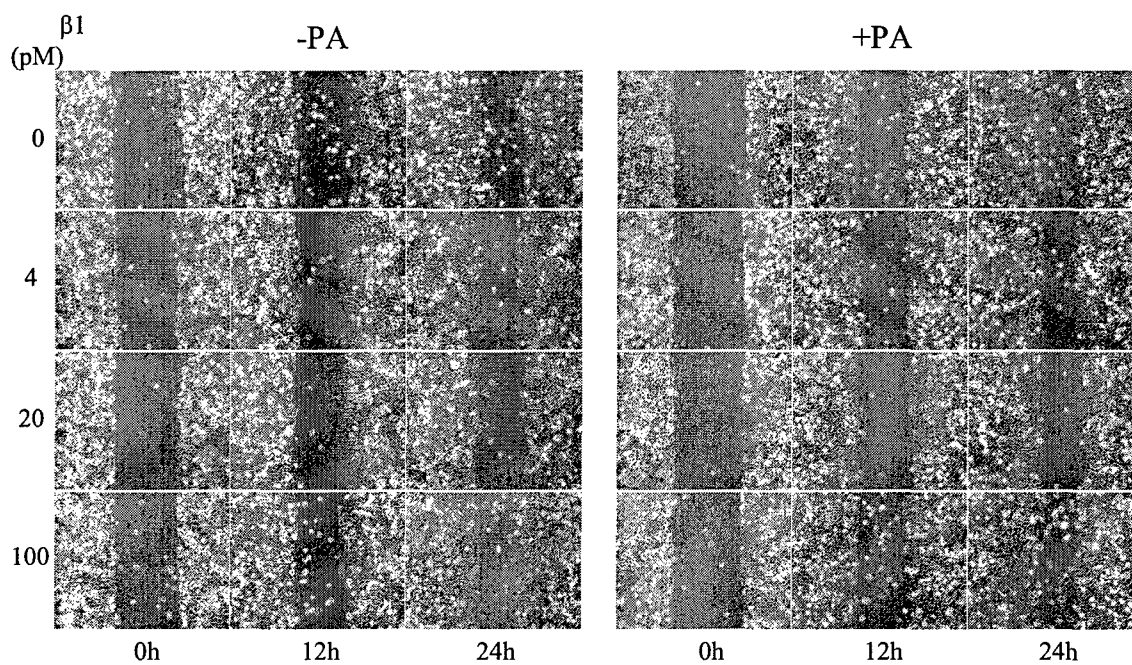


Fig. 8